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THE LABORATORY PROPAGATION OF *SPIROMETRA MANSONOIDES* (MUELLER, 1935) AS AN EXPERIMENTAL TOOL. III. IN VITRO CULTIVATION OF THE PLEROCERCOID LARVA IN A CELL-FREE MEDIUM

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As previously reported in abstract (Mueller, 1958), the procercoid of *Spirometra mansonoides* has been grown in a cell-free medium to the plerocercoid stage, in the complete absence of the second intermediate host from the life history. Such cultured plerocercoids regularly produce infection with the adult worm when fed to cats (Mueller, 1959c). Since the medium employed contains only standard tissue culture ingredients, the problem of culturing these larvae, once solved, turns out to be one of laboratory manipulation, rather than of satisfying any unusual metabolic requirements on the part of the worm.

Previous papers have described the technique of collecting and hatching the ova (Mueller, 1959a) and of propagating and infecting the copepod host in laboratory culture, and harvesting the procercoid larva in mass (Mueller, 1959b). The axenic procercoid, collected by the method described, forms the starting point for culture. This paper will describe the technique of maintaining and manipulating these cultures.

CULTIVATION OF THE PLEROCERCOIDS

Preparation of medium and materials

The usual routines of tissue culture are observed throughout: sterile technique, special washing of glassware, use of demineralized water, precaution against deterioration of materials, and the like. Most materials and prepared media are stored in the cold room at 4° C. Blood serum and excess chick embryos are frozen and stored at – 20° C. Preparation of medium is conducted as far as possible in the cold, or by keeping containers chilled in pans of cracked ice. A standard tissue culture manual, such as Hanks, 1955, should be consulted for information on these procedures.

The essential ingredients of the medium are Mixture 199, calf serum (CS), and chick embryo extract (CEE). For preparing CEE, Hanks' balanced salt solution (BSS) is used. Mixture 199 and calf serum are purchased from Microbiological Associates, Inc., Bethesda, Maryland, and Hanks' balanced salt solution from Difco Laboratories, Detroit, Michigan. Mixtures 199 and BSS are purchased in 10× concentrates. CEE is prepared from 11-day-old chick embryos. Embryonated eggs are delivered to the laboratory once a week, in 4-dozen lots, by a local poultryman,

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and incubation is continued in the laboratory. Development is timed so that 2 dozen eggs are 11 days old on Tuesday, and 2 dozen on Friday, of each week.

Hanks' BSS is prepared by mixing 20 ml of the concentrate with 180 ml of demineralized water, and bringing the pH to 7.7 with 10 percent $NaHCO_3$. Medium is prepared twice a week in lots of 50 tubes (100 tubes per week). This is sufficient to take care of about 10 cultures running simultaneously, all of them changed daily, and the more active twice daily. Chick embryos are harvested in the regular manner, rinsed in BSS, measured in a 50-cc syringe, used as a graduate, placed in a Waring Blendor, previously chilled, with an equal quantity of BSS, and a sufficient amount of Combiotic (buffered crystalline, Pfizer) to give 500 units of penicillin, and 600 mcg streptomycin per ml. The Blendor is run for 2 or 3 minutes. The mixture is transferred to wide centrifuge tubes and spun in the cold room at 2000 RPM for 15 minutes. The supernatant fluid is then filtered through glass wool for further clearing. This is accomplished by lightly twisting a plug of glass wool (Corning No. 790) around the point of a fine forceps, and inserting into the neck of an 80-mm funnel fitted into a filter flask. Practice is required to make a plug of the proper density, and the filter must be washed with a liberal quantity of water to avoid glass splinters in the medium. The apparatus is then wrapped and autoclaved.

Mixture 199 is made by bringing together 30 ml of the 10× concentrate, 30 ml of calf serum, and 240 ml of demineralized water. Combiotic is added to give 500 units of penicillin, and 600 mcg streptomycin per ml. The pH is adjusted to 7.7 with 4.4 percent $NaHCO_3$. The mixture (199-CS) is then tubed, 6 ml to the tube, in Kimax screw cap (rubber lined) culture tubes, 16 mm × 125 mm, 16 ml capacity. To each tube is added 1.5 ml CEE, making 7.5 ml of medium per tube.

Several racks of similar screw cap tubes are kept on hand, some empty, some with physiological saline, 8 ml to the tube, and several dozen tubes of 199-CS, 6 ml to the tube. Additional antibiotics are prepared as follows. One or 2 drops of Kantrex (Bristol Laboratories brand of kanamycin) are added to a tube of 199-CS. A solution of Acti-dione (cycloheximide crystalline, Upjohn) is prepared in a screw cap bottle, 1 gm crystalline substance to 100 ml distilled water (10,000 mcg/ml). Mycostatin (Squibb) is an almost insoluble powder. If the crystals come in contact with the worms they have a corrosive, lethal effect. To avoid this difficulty, a saturated solution is prepared by adding several drops of the concentrated Mycostatin to a tube of 199-CS, so that excess crystals lie on the bottom. The clear solution is drawn off the top, as needed.

Special laboratory facilities

As in all tissue culture, the periodic inspection of cultures, and replacement of exhausted medium, without loss of worms, or introducing contamination, presents a problem. This has been solved as follows. An AO Spencer series 23LF stereoscopic microscope, with 1×, 2×, 3× objectives, and 10× inclined eyepieces, is clamped into the position indicated in figs. 1 and 2. An unsilvered right angled prism (figs. 3 and 4), 1½ inches square on the sides forming the right angle, is provided with a short length of mm ruler, and a protective glass cover cut from a microscope slide. The ruler, face down, and slide are attached to the upper surface of the prism by narrow strips of masking tape. The prism (figs. 1 and 2, p) is supported in a frame

so that the upper face is 11 inches above the table level. The support actually in use, and figured, consists of a large slide box, a wire basket, and a small slide box, surmounted by a wedge to hold the prism, the whole taped and wired together. A loop of wire passing over the small slide box in front of the prism serves to engage its lower edge and keep it vertical. The prism is backed by a strip of black velvet (bv), which hangs loose in front. This flap is folded up over the prism to form a dust cover when not in use. A small sterile transfer room is fitted as indicated in figures 1 and 2. A large ring stand (the type figured is Labline, Inc.) stands behind the prism base, and is taped to it, and both are pushed back against the bench fixtures for further rigidity. This ringstand (rs) supports a clamp overhanging the prism, and a goose neck lamp from which the base has been removed. Large and small Bunsen burners (b1, b2) are placed as shown, the smaller elevated on an inverted wire basket for greater convenience in grasping. An additional ring stand is located on the left, supporting a wooden test tube clamp and beaker of tap water (b). The test tube clamp is used to hold the Pasteur pipette (pp) when not in use. The pipette forms the operational end of the aspirator, and is connected to the vacuum port (v) by a long gum rubber tube, ⅜ inch external diameter, and trap. The trap (t) consists of a filter flask, held between the nozzles of the air outlet (a) by a wide rubber band, and connected to the vacuum port by a short length of pressure tubing. A pan (pa) for discarded tubes completes the arrangement of the bench. A special valve (va) for the vacuum line is installed on the front of the bench so as to be conveniently accessible to the left hand of the operator.

A light hospital cart, 20×36 inches, 33 inches high, with racks for the cultures, new medium, physiological saline, antibiotics, and cans of Pasteur pipettes, cotton plugged and plain, and a diary calendar for notes, stands to the left of the operator during transfers. A roller drum is located in an incubator room, maintained at 37° C. The drum makes one revolution in 5 minutes, and is set at a somewhat steeper angle than usual because of the large volume of medium in the tubes.

Management of cultures

A harvest of procercoids, several thousands or more, (fig. 12) obtained as described in part II of this series, is drawn off into a sterile screw cap tube, with about 6 or 7 ml of 199-CS, and allowed to settle. The tube is placed in the clamp above the prism (figs. 1, 2, 4) and inspected through the binocular microscope, with the objective in the lower position indicated on fig. 4. This gives a view up through the bottom of the tube (figs. 6, 7, 8). Any of the 3 objectives can be used. For determining size of the procercoids, the clamp is loosened so that the tube can be slanted slightly to overlap the scale. The procercoids are from 0.1 to 0.3 mm in length, and almost entirely free of foreign material (fig. 11) if the procedure has been conducted properly. The 199-CS is now replaced with freshly prepared 199-CS-CEE, and the tube placed in the roller drum. It is examined at least twice a day, and medium replaced at least once daily. The worms grow rapidly at first, almost doubling their size in 24 hours, and some attain a length of 1 mm in one week. The cultures quickly become crowded, necessitating removal of some of the worms, or division of the cultures. The best growth obtained to date is 10 mm in 28 days. During the early phase of growth, the pH of the medium changes rapidly, falling from 7.7 to 6.5 in 24 hours. Hence in the more crowded or active cultures medium

is changed every 12 hours. After a length of 5 to 7 mm is reached, however, metabolism seems to slow down markedly, with the pH dropping only to 7.2 or 7.3 in 24 hours. Whether this represents a true change in metabolic rate with age, or whether it is a dilution effect has not been determined. As the worms increase in size, the number of worms per tube has to be greatly reduced. The most active part of the worm is the scolex. Small spargana are all scolex, whereas larger worms are mostly relatively inert tail.

Observed in culture, the worms move more or less continuously. Procercoids periodically elongate and contract. But by the end of the first week, the wave-like peristaltic movement characteristic of the sparganum sets in. Well-defined bothria are visible during the second week. There is an enormous variation in growth rate of different worms in the same tube, and often between different cultures of identical origin. Some worms fail to grow. Whether this is due to crowding and competition, or to some inherent difference in the worms remains to be determined.

After the worms have attained some size, a flocculum of precipitate forms a collar around the neck, and plugs the bothria (fig. 9), as though these surfaces were intensely interactive with the medium. This precipitate clouds the wall of the tube, interfering with observation, and also hastens the deterioration of the medium, so that eventually it must be removed. Once a week, therefore, the worms are washed and transferred to new tubes (see below).

In the initial stages of culture a certain number of tubes exhibit contamination, carried over from the copepods. If this is bacterial, one or two drops of kanamycin, previously diluted (see above), are added to the tube; if filamentous mold, from 1 to 4 drops of Acti-dione solution (10,000 mcg/ml) are added. If yeasts are present, from 5 to 10 drops of Mycostatin are added. If several treatments fail to clear the culture, it is discarded, since continuous use of these agents retards growth and injures the worms. The loss of several cultures in a series is of small importance, since the necessary thinning of those that remain more than replaces the loss. It is most convenient to run at least 10 cultures at a time.

Renewal of medium

As previously stated, this must be done at least once a day, or oftener in the case of very active cultures. Culture tubes are removed from the roller drum, placed in a rack on the cart, along with other equipment itemized above, and wheeled into the sterile transfer room. The valve (v), figure 1, is permanently opened to give a strong suction. The accessory valve (va) is used only for on and ff. A culture is taken from the rack, the cap loosened, and the tube placed in the supporting clamp above the prism. The cap is now removed. The small burner (b2) is used to flame the mouth of the tube. The cap is replaced, but without screwing down. The pipette is dipped into the beaker (b) several times, while the valve (va) is opened to flush it out. The valve is then closed and the pipette lightly flamed by passing several times lengthwise through the burner (b1). The valve is then opened, the cap lifted from the tube, and the pipette is lowered into the tube. The changing tone made by the suction of the pipette as the meniscus falls gives auditory information on the position of the tip, while the worms are watched through the microscope. The binocular may be used in either of the positions indicated in figure 4, the upper

being the best for controlling aspiration, while the lower is better for observing the culture. The medium is sucked up almost as a vapor, with minimal disturbance of the worms. In this way a tube can be aspirated almost totally without loss of any of the larvae if that is desired. This detailed control is particularly important in young cultures with very small worms. In older cultures the process can be controlled with the naked eye.

After the old medium has been removed, the pipette is dipped into the beaker several times to flush the system, and then placed in the support. The mouth of the tube is again flamed, and the cap replaced and lightly screwed on. The tube is removed from the clamp, and may be refilled at once, or placed in the rack until the other cultures have been aspirated. To refill, a culture is taken with the left hand, and a tube of medium with the right. By a familiar operation, the caps of the tubes are removed with, and held in, the opposite hands. The mouths of the tubes are lightly flamed in burner b1, and the medium transferred by pouring. With a little practice, this can be quickly done with no spillage on the outside of the receiving tube. The contents of the right hand (the new tube and old cap) are dropped into the pan. The culture tube is flamed and the new cap screwed on. Thus at each change of medium, the old cap, the part of the system most vulnerable to contamination, is replaced with a new and sterile one. It is not necessary to warm the new medium to room temperature before use, since the worms are tolerant of temperature shock. But tubes must be tightly capped before replacing in the incubator to prevent loss of CO_2.

With this system it is easy to change 10 cultures in 15 or 20 minutes. The pipette is flushed and flamed to steaming before each transfer. The vacuum must be turned off during flaming to avoid sealing the tip. A single pipette can be used for many transfers, at a considerable saving of glassware. But it should be periodically replaced, since a charred scum eventually accumulates on the inner surface.

Worms accidentally or purposely aspirated are caught in the trap, and can be easily retrieved by the apparatus shown in figure 5: a 4-inch funnel, fitted with a short length of Tygon tubing, and a spring clamp. The contents of the trap are poured into the funnel. Worms rapidly settle in the stem, and can be removed with a few ml of liquid for study or fixation.

Cleaning of cultures and changing tubes, to get rid of protein precipitates is done once a week. The old medium is drawn off. A tube of saline is poured into the culture and immediately poured back into the saline tube. With a little manipulation, and several pours back and forth, all the worms can be transferred to the new tube. The worms rapidly sink, and the saline is drawn off while the flocculum is still suspended. By pouring more saline into the tube to agitate the contents, and aspirating several times, the worms can be effectively cleaned. The process is of course controlled through the microscope. Trypsin may also be used to clean cultures. But the method given is simpler. These procedures are described in detail, because they have been evolved only as the result of much experience. While they may seem complicated, they are absolutely minimal for efficient operation. Their exact observance spells the difference between success or failure. By these techniques I have been able to keep cultures for 90 days, with many changes of medium and transfers, and end with axenic worms, as verified by direct examination and by culture methods.

INFECTION OF CATS WITH CULTURED SPARGANA

Cultured spargana, of procercoid origin, from 3 to 10 mm long, have on various occasions been fed to worm-free cats, and all of 15 cats employed became positive for ova. The prepatent period varied from 10 to 18 days, with an average of 12 days. Worms recovered from these cats have normal morphology. Ova have been collected, hatched, and copepods infected with the coracidia. The infected copepods have produced heavy sparganum infections when fed to mice. I have also harvested procercoids from such copepods, and grown a second generation of spargana in vitro, and with these infected cats a second time. These second-generation, cultured spargana, like the first, also show great variation in size and rate of growth. Whatever the factor responsible for this variation, it is obviously not eliminated by one passage through culture. Cultured sparganules also produce infections and grow into large worms, when injected intraperitoneally into mice. There thus appears to be no qualitative difference between spargana reared in culture and those grown in the mouse or other normal intermediate host.

CULTURE OF SPARGANA OF MOUSE ORIGIN

When this work was begun early in 1956, the logical place to start seemed to be the familiar sparganum as recovered from experimental mice, and much time was spent trying to grow these in vitro. Large worms were decapitated, and the rugged larval scoleces, measuring from 3 to 5 mm in length, were placed in tissue culture. At first Earle's medium was used, and later 199-CS, each of these successively modified and supplemented in various ways, and submitted to a variety of temperatures and atmospheric partial pressures (anaerobic and CO_2). Although the worms survived up to periods of from 9 months to 1 year, and at the end of this time could be regenerated by injecting into mice, growth in vitro could not be demonstrated. Some elongation of the body occurred, but obviously only by exhaustion of tissue reserves, and the worms became thin and transparent.

It was not until approximately a year after culture work was begun that CEE and procercoids were used for the first time. The rapid growth of procercoids in 199-CS-CEE was so dramatic that all effort was soon concentrated on these. When CEE was added to the 199-CS medium containing 9 and 10 month old scoleces of large decapitated spargana, it produced a startling effect. The tip of the head grew out to a "needle nosed" affair in 24 hours. Then the worms died as though the effort had exhausted the starved tissues. When freshly decapitated scoleces from large spargana are placed in 199-CS-CEE, no such effect and no demonstrable growth occur. This is reminiscent of cultures of procercoid origin, since in these, too, growth seems to slow down markedly after a length of 5 to 7 mm is reached. Why CEE should stimulate a "starved" scolex, and not a fresh one, is a provocative question.

Before the technique of mass harvesting of procercoids, described in part II, was worked out, they were dissected from copepods under the microscope with *minuten Nadeln,* and my first procercoid cultures were started with such material. But the labor involved, and the frequency of contamination, plus the small numbers obtainable by such methods, deprived such cultures of any practical value as an experimental tool. Other methods were called for.

When a culture of infected copepods is fed to 10- to 15-gram mice, the procer-

coids penetrate the intestinal wall in from 5 to 10 days, and break out into the abdominal cavity. These sparganules are from 0.5 to 1.0 mm in length. They flourish in culture, and perhaps represent the easiest way of establishing axenic cultures.

The following technique is recommended for collecting such sparganules. Infected mice (5 to 7 days from date of feeding) are lightly etherized. A fold of skin is picked up over the back, and snipped with scissors. With one quick movement, the skin is stripped both ways to expose the carcass. The heart is still strongly beating. The neck is severed with one stroke of the scissors, and the animal quickly exsanguinates through the carotids. The legs and tail base are then snipped off to get rid of the skin. The abdominal wall is still intact. The carcass is rinsed under the warm-water tap to free it of blood, and placed in a dish of physiological saline. The body cavity is opened with scissors, and the muscular belly wall cut away, so that the mesenteric loops float freely. The intestine is intact. Several mice can be quickly prepared in this manner and placed in the same dish. The dish is warmed gently with an overhanging lamp. Since the animals have been thoroughly bled, there are few blood cells to cloud the saline. After 15 minutes, the carcasses are removed, after gentle washing with a pipette. Excess saline is poured off, and the dish searched under the microscope. Large numbers of the tiny white worms will be found on the bottom, where they can be concentrated by rocking, picked up with a fine pipette, and removed to a tube of saline or 199-CS for washing. They are then placed in culture. Initial contamination of such cultures is negligible compared to procercoids harvested from copepods.

DISCUSSION

Many workers have attempted to grow tapeworms in culture, but until Rausch and Jentoft (1957) reported their work on *Echinococcus multilocularis,* no genuine success was had. Von Brand (1957) pointed out that Smyth's work on inducing maturation of certain pseudophyllidean plerocercoids (*Ligula, Schistocephalus*) in vitro, could not be considered true growth, since "these tapeworms are already far along in their development when removed from the intermediate host," and sexual differentiation is not accompanied by any increase in mass. Neither can Smyth's (1956) claim for somatic mitoses "as a criterion for assessing growth in vitro" of *Diphyllobothrium* sp. be accepted, in the absence of other evidence. The words of Kimura (1953) are here quoted: "It is a fact that tissue cells can grow to some extent in Ringer's solution, Tyrode's solution, or physiological saline. Multiplication under these conditions must be considered a process of survival on the part of the cells, and is merely a process based on the use of their own nutrients. This is not usually regarded as tissue culture in the true sense of the word." What is true for tissue culture applies with even greater weight to the culture of such an organism as a parasitic worm.

Through the work of Dévé, Coutelen, and others, it has long been known that scoleces of *Echinococcus granulosus* can be maintained in vitro, in a mixture of blood serum, ascitic, and hydatid fluids, if the medium is bacteriologically sterile, and that some scoleces will vesiculate and produce a laminated membrane. This has been interpreted as growth, although it is small in degree. While the work of Rausch and Jentoft is more convincing, *Echinococcus multilocularis* leaves something to be desired as an experimental organism. It is inert and small in dimension,

and the vesicular habit implies small mass. It is slow growing, and prospers only in a medium containing HeLa or other cell lines. Although such cultured vesicles produce infections when injected into voles, infection of the dog with the adult worm from cultured material remains to be demonstrated.

Spirometra mansonoides has long seemed to the author a good object for such studies. It uses common hosts so that all stages can be readily propagated in the laboratory. The sparganum naturally occurs as a tissue parasite under conditions which have been successfully duplicated for many tissues, so that the worm should be susceptible to culture by similar methods. Parasitic in both warm and cold blooded hosts, and in all classes of vertebrates except fish, it is obvious that the larva has a wide range of temperature tolerance, and an almost total lack of host specificity. During life the larva is constantly moving, in a characteristic fashion, so that one can determine at a glance the vitality of the cultures, and to some extent the condition of the worms. The success of the work reported bears out the correctness of this original conjecture.

Archer and Hopkins (1958) writing on the in vitro cultivation of helminths, state: "One of the greatest difficulties in this line of work is the selection of criteria to determine whether the worms being cultured are developing normally," and they propose a series of biochemical studies (on a species of *Diphyllobothrium* from the whitefish, maturing in the laboratory rat) to establish a base of comparison for such studies. Smyth (1955) stressed the distinction between "growth in vitro as opposed to mere survival," laying particular emphasis on the ability of cultured larvae to differentiate, mature sexually, and produce fertile eggs, capable of repeating the life cycle, as evidence of true culture. Since my cultured larvae have been carried through the remainder of the life cycle in normal hosts, and again cultured, it is obvious that Smyth's postulates have been met, as far as the sparganum is concerned. And such basic biologic requirements having been fulfilled, any appeal to biochemical criteria is superfluous

The medium described in this paper is merely the first one hit upon which gives positive results. Further work is required to determine to what extent it can be simplified or improved, and to elucidate and define the specific nutritional requirements of the worm.

SUMMARY

1. Procercoids from infected copepods have been grown in a cell-free medium, from a length of approximately 0.1 mm to 10.0 mm in 1 month, and to 20 mm or more in 2 or 3 months. This represents an increase in mass of several thousand percent.

2. The medium consists of Mixture 199 with 10 percent calf serum, to which chick embryo extract (11-day embryos) is added in the proportion of 6 ml of 199-CS to 1.5 ml of CEE.

3. There is great variation in the growth rate of different cultures, and in different worms in the same culture.

4. Culture-reared spargana mature when fed to cats.

5. Ova from worms in such cats hatch normally, and are capable of repeating the life cycle in all the normal hosts. The plerocercoid has also been reared in culture for the second generation, and a second series of cats infected from such worms.

6. All criteria of true culture, as opposed to mere survival, have been met.

7. Apparently the culture system described can substitute for the second intermediate host indefinitely.

8. The worms grow most actively during the first few mm of development, practically doubling their size in the first 24 to 36 hours.

9. Growth of worms, and utilization of medium slows down markedly as the worms attain a length of 5 to 7 mm. This may be a dilution effect, since the cultures are thinned out as the worms increase in size. The most active part of the worm is the scolex. Small worms are all scolex, whereas large worms are mostly inert tail.

10. Precipitates form around the head and neck of the worm, suggesting intense interactivity between medium and this region of the worm.

11. The cultures are axenic.

12. Simple and efficient methods are described for observation and manipulation of the cultures.

ACKNOWLEDGMENTS

Over the several years these studies have been in progress, many people have assisted the author in various phases of the problem. I am particularly indebted to those who taught me the rudiments of tissue culture. Mr. Ory Carbonero first suggested the use of Mixture 199 and calf serum, and Dr. Carl Silver prepared my first chick embryo extract. At various times Miss Marjorie Smith, Mr. Robert Buck, and Mrs. Lucy Bliemel have been in charge of media and glassware. Miss Winifred Osborne, of the Syracuse City Laboratory, has advised on antibiotics, and various problems connected with tissue culture. The work has been assisted by grants from the State University of New York Research Foundation, and the National Institutes of Health.

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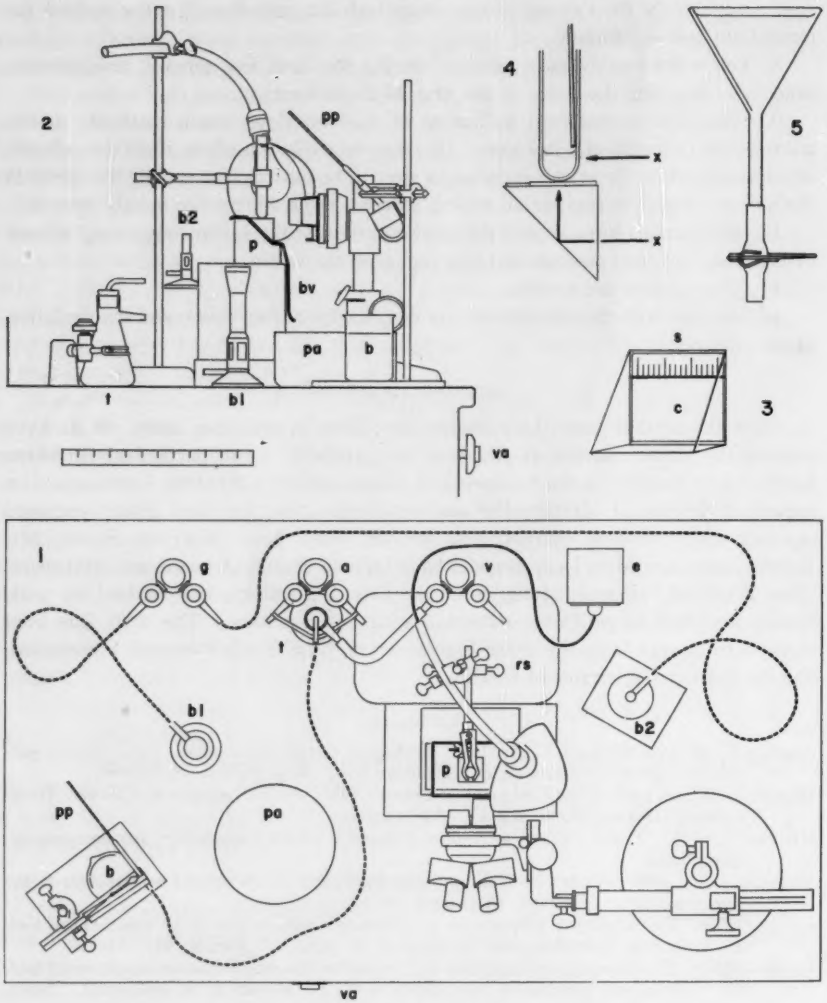
PLATE I



EXPLANATION OF PLATE I

Scale between figures 1 and 2 represents 1 foot, divided in inches, and applies only to figures 1 and 2.

FIGURES 1 and 2. Plan and side view of laboratory bench in dust-free cubicle, showing equipment for observation of cultures, and renewal of medium. Hose connections are indicated by dotted lines. In addition to equipment shown, a small cart with various items stands to the left of the operator during manipulations. See text.

FIGURE 3. Prism assembly, viewed from below, consisting of a 1.5-inch right angle unsilvered prism. Near the rear edge a 30-mm scale (s) is fastened face down (only every second mm is indicated on the drawing). In front of this is affixed a glass cover (c) to protect the prism.

PLATE II

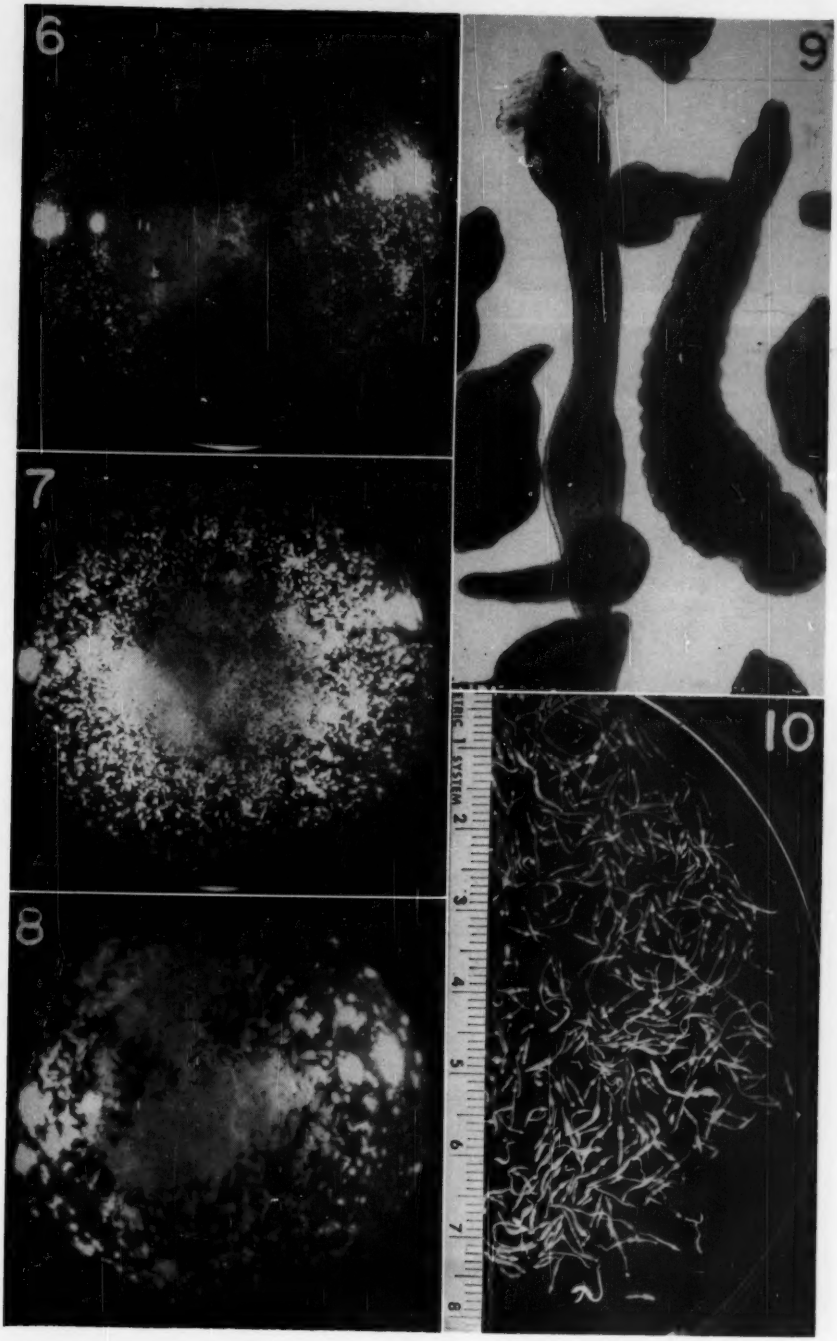


PLATE III

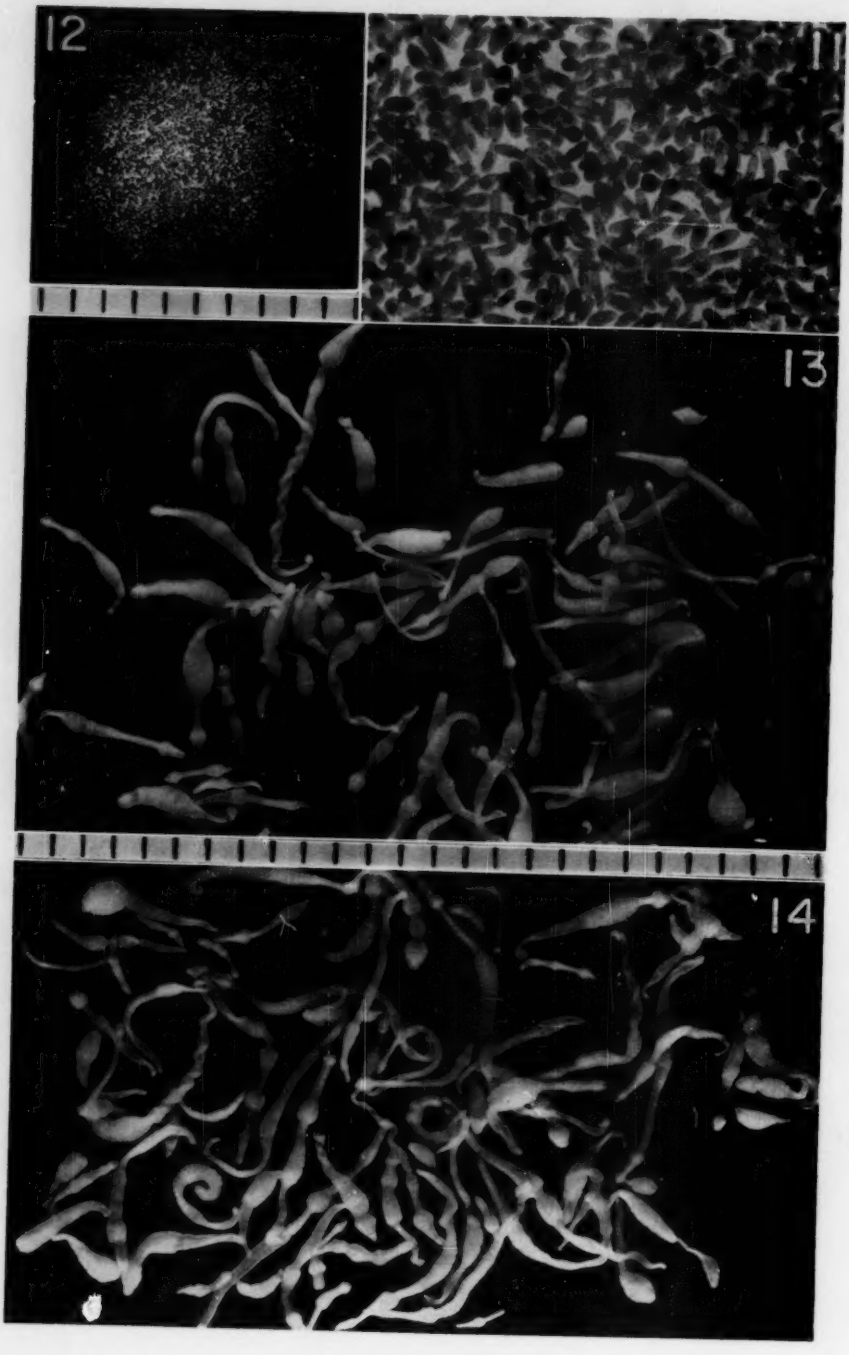


FIGURE 4. Side view of prism and culture tube with aspirator tip in position. X, X represent alternate lines of sight for the microscope objective. The adjustments of the microscope have sufficient range to permit easy shifting from one position to the other.

FIGURE 5. Worm retriever. A 4-inch funnel, fitted with a length of Tygon tubing and pinchcock, and supported on a ring stand. (See text.)

EXPLANATION OF PLATE II

Figs. 6, 7, 8 are photographed through the prism, showing the bottom of the culture tube and worms as seen in the normal position of viewing. The scale with figure 10 applies only to that figure.

FIGURE 6. Culture F-100a, 3 days old.

FIGURE 7. Culture F-94a, 7 days old.

FIGURE 8. Culture F-92b, 17 days old.

FIGURE 9. Culture F-92b, 26 days old. Note plugs of precipitate in bothria of one worm.

FIGURE 10. Culture F-99, 67 days old. Photographed in petri dish. Many worms are 10 mm long. This was a primary culture, and too crowded for maximum growth.

EXPLANATION OF PLATE III

Scales, in mm, apply to figures 12, 13, 14; not to figure 11.

FIGURE 11. Procercoids, seen with transmitted light, showing numbers obtained by method described in part II of this series, and freedom from extraneous matter. These were harvested after 11 days in the copepod, are infective, and will grow in culture.

FIGURE 12. Procercoids, photographed with dark background. Average inoculum used to start a culture. Photographed with identical set-up and magnification used for figures 13 and 14.

FIGURES 13 and 14. Portions of culture F-106.1.2, at 33 days of growth. Several worms are 10 mm long. This culture represents the second generation of spargana raised in vitro, without benefit of the second intermediate host. Note variation in size of different worms.

RESEARCH NOTE

CUTICULAR PEELING: A DISSECTION TECHNIQUE FOR PREPARATION OF CESTODE WHOLE MOUNTS.

Clear whole mount preparations of thick or old preserved tapeworm specimens can result from the peeling or shredding of the cuticular and outer muscle layers under a dissecting microscope. The method herein described was developed by Evelyn K. Hake, research assistant to the late Dr. Asa Chandler, and has been used with considerable success in this laboratory. The technique is both simple and flexible, requiring only a fine, specially ground scalpel or flattened needle, jeweler's forceps, and a steady hand. Cestode proglottids can be dissected in terpineol after completion of staining and dehydration. This clearing solution is a softening agent which renders the material easier to work with. Xylol-diluted balsam can also be used, although this mounting medium has the disadvantage of eventually drying out and turning acid in older preparations. Terpineol, used as clearing agent and dissection medium, followed by mounting in heated, natural (undiluted) Canada balsam, have proved to be the best combination.

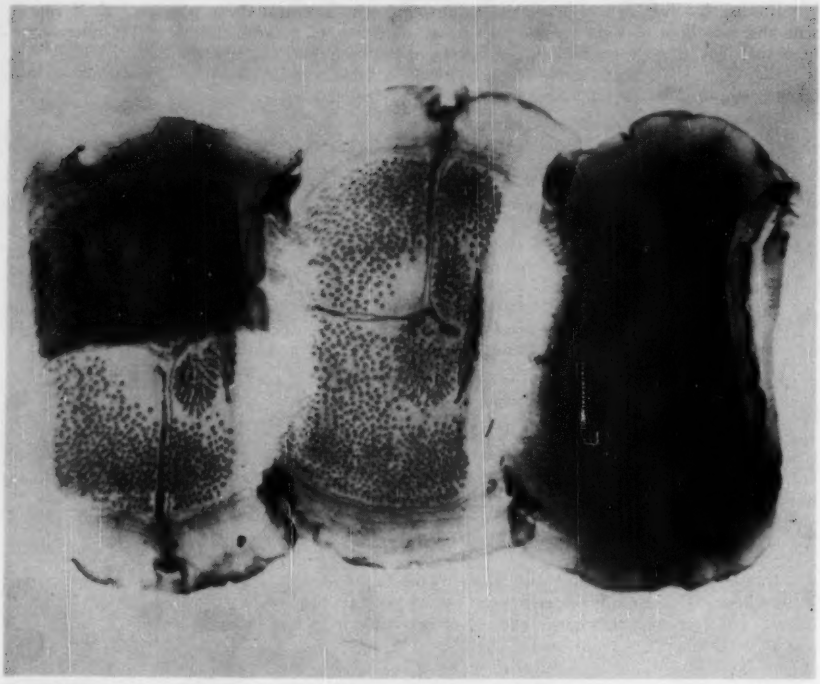


FIGURE 1. *Taenia saginata.* Mature segments showing effect of removal of cuticle and surface muscle fibers from both dorsal and ventral surfaces (center), from dorsal and ventral portion of one side only (left), and an intact segment (right).

Stripping the muscle sheath from the tapeworm segments is facilitated by the layering of the muscle cells and the anteroposterior orientation of the fibers. With practice, one can strip off the dorsal and ventral layers in a number of discrete fiber bundles, leaving only the central parenchyma with the reproductive organs. Figure 1 shows a group of mature *Taenia saginata* segments from a folded, thick specimen kept for many years in formalin. The proglottids were partially separated and the specimen was about to be discarded. Yet careful application of this technique turned it into an excellent preparation, showing the reproductive system and excretory ducts in striking clarity. Similar preparations can be made from gravid segments.

Modification of the procedure can be used to partially dissect helminths or to remove the muscle layer from one surface only. Trematodes are more difficult to work with, but thick specimens can be cleared of parenchyma or eggs from specific portions of the worm. Often a whole mount dissection of the reproductive complex will prove more useful and is simpler to interpret than are sectioned specimens. For students' use, alternate peeled and intact segments (as in fig. 1) can be prepared for a single slide, giving the student a better concept of the reproductive organs within the parenchyma and of the enveloping sheath of muscle tissue and cuticle.

Thanks are expressed to Mrs. Grace Hsu for preparation of the specimen shown and to Dr. Richard A. Boolootian for taking the photograph.

(This study was supported in part by ONR Contract NR-103-446.)—DONALD HEYNEMAN, *Department of Zoology, University of California at Los Angeles.*

THE HEXACANTH EMBRYO OF A DILEPIPID TAPEWORM. II. THE EPIDERMAL GLANDS AND POST-MATURATION CHANGES

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Interesting granular epidermal glands are characteristic of cyclophyllidean oncospheres of the hymenolepid-type (Ogren, 1957). These glands, first described by Reid (1948), are found in *Choanotaenia, Hymenolepis,* and *Raillietina cesticillus.* They are also present in *Dictymetra, Diorchis, Protogynella, Oochoristica,* and *Dilepis* (Ogren, 1953, 1958a). Probably all true genera in the families Hymenolepididae, Dilepididae and Linstowiidae will be found to have oncospheres with epidermal glands.

Development of the gland area is similar in *Hymenolepis nana, H. diminuta,* and *Oochoristica symmetrica.* The gland differentiates from 2 bilateral primordia, that enlarge, spreading over the embryo to form a U-shaped area of several cells (Ogren, 1955, 1957, 1958b). The following study of *Dilepis undula* Schrank confirms this pattern.

The hexacanth embryo has 4 overlapping periods of differentiation: (1) Morula formation; (2) hook and parenchyma development; (3) epidermal gland development; (4) post-maturation changes (Ogren, 1957). These periods are identifiable in *Dilepis undula.* The first 2 have been considered somewhat in Part I (Ogren, 1958a) along with methods. The present part combines description of epidermal glands and post-maturation changes.

Development and Appearance of the Gland Area in Dilepis

Glands, not recognizable in the morula, are first evident as bilateral primordia during early hook formation (figs. 5, 6). The 2 lateral blastomeres in each primordium (figs. 2, 5) are characterized by prominent interphase nuclei and distinct cell membranes. The cytoplasm contains abundant closely packed granules, which give a positive ribonucleic acid reaction with pyronin or toluidine blue. The primordia stain pink, or slightly acid, with neutral red in vivo. These largest and most characteristic cells of the embryo, in subsequent development, form 2 lateral comma-shaped areas (fig. 7).

A mature U-shaped gland is formed in oncospheres with fully developed hooks, by the joining of these cells through a narrow isthmus (fig. 10). The gland develops dorsal and ventral portions with arms extending forward, while a granular cover forms over the region behind the hooks (fig. 8). The gland tends to spread over the oncosphere by the joining of cells. Cell boundaries are obscured (fig. 10), although several nuclei can be identified in each portion. Secretory pores and ducts are not evident. The terminal parts of the arms end blindly in non-granular protoplasm associated with the cortex (figs. 8, 9, 10). Secretion is believed to pass through the surface of the entire gland. This explains the adhesive body of these oncospheres and the origin of large thin-walled vesicles attached to the cuticle.

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Mature epidermal gland cells, with large interphase nuclei (fig. 1), have masses of Schiff positive material along the inside of their cell membranes. The cytoplasm is often quite fluid and contains many large granules. In fixed material the mature granules are eosinophilic, metachromatic, Schiff positive, and no longer stain with pyronin. The cells give an alkaline, yellow-to-orange reaction with neutral red in vivo. The cells often appear as sacs filled with large granules showing agitated motion. The granular protoplasm flows forward toward the hooks during extension of the oncosphere. During retraction the granules flow in the opposite direction.

Post-Maturation Changes in the Oncosphere of Dilepis

Oncospheres from terminal proglottids show post-maturation changes already recognized in other cestode embryos (Ogren, 1956, 1957). These changes resemble those of cyst and spore formation. They are as follows:

(1) The oncospheres appear more compact, with dense muscular parenchyma and thick cuticle. The hooks are retracted into the central part of the body at a characteristic angle (figs. 11, 12).

(2) The prominently stained gland occupies a large part of the body and has a nearly closed C-shape (fig. 12). The broadest, most intensely stained portions are near the hooks and result from folding of the arms. Glands in active oncospheres, free from the shell, have a typical U-shape (fig. 9). They now stain more intensely with all the counterstains; e.g., dark purple with toluidine blue and red with safranine O or eosin, in comparison with earlier reactions.

(3) Deposits of pyronin-stained ribonucleic acid (Ogren, 1952) accumulate around certain nuclei, especially in gland cells (figs. 3, 4, 9, 12). In other oncospheres, this is characteristic of RNA-rich plastin cells with embryonic potencies. Neutral red stain used on live oncospheres colors the dense RNA red and the granules yellow (figs. 11, 12).

(4) In the oldest oncospheres, parenchymal nuclei tend to shrink, become elongate, stain darker than previously, and develop a thick membrane.

(5) Metachromatic and Schiff positive substances accumulate in the oncosphere and in the region between the embryonic membranes. These reactions are destroyed following digestion with saliva at 40° C for 40 minutes, indicating a polysaccharide such as glycogen is present.

DISCUSSION

There are two kinds of embryonic glands, or secreting areas, in oncospheres: (1) Epidermal glands, present in *Dilepis, Oochoristica,* etc.; (2) mesenchymal glands, present in *Taenia* (Silverman, 1954; Silverman and Maneely, 1955), *Mesocestoides* (Ogren, 1956), and other similar embryos, composed of 6–40 RNA-rich mesenchymal plastin cells occupying the major area behind the hooks. Both kinds of structures have been called penetration glands in the literature. Their functional relationships are not well known. The original suggestion by Reid (1948) that the secretion aids in penetration of intestinal epithelium is widely accepted. Additional analysis is presented in my study of *Oochoristica.* Secretions of embryonic cells in the oncosphere may possess one or more of the following: (1) Cytolytic ability; (2) anti-clotting, metachromatic, heparin-like property; (3) adhesive property;

(4) colloidal-gel property, forming membranes around the oncosphere (Ogren, 1955, 1957). The gland also persists for a time after the oncosphere enters the tissue of the intermediate host, and may have a protective function at this time (Ogren, 1957). Available evidence demonstrates, however, that gland cells along with other embryonic cells become transformed into RNA-rich plastin cells. These are the embryonic cells of the metacestode and under various circumstances are secretory, contractile, or germinative (Ogren, 1953, 1956, 1957).

The post-maturation period is difficult to explain. It may represent changes to protect the embryo when in adverse environments, prepare it for penetrating the alimentary tract of the intermediate host, and prepare for metacestode differentiation. Glycogen, lipid and phospholipid are present in post-maturation oncospheres of *Hymenolepis diminuta* and *Raillietina cesticillus* (Hedrick, 1958; Hedrick and Daugherty, 1957). Polysaccharide substances are present in the gland areas of *Taenia* (Silverman and Maneely, 1955).

Periods in the development of the hexacanth embryo may show characteristic metabolic activities. One may predict that these will be reflected in the regional metabolism of the strobila. Some evidence for this has been presented by Read (1956) and Daugherty and Taylor (1956).

SUMMARY

In *Dilepis undula* Schrank, the epidermal glands, located in the cortex of the oncosphere, are restricted largely to the region behind the hooks. Gland primordia are present in embryos before hook blades are completed. The large granules characteristic of these cells stain prominently with cytoplasmic stains. Their acidophilic and metachromatic properties increase in the post-maturation condition. Gland nuclei become enclosed by a dense region of ribonucleic acid. Post-maturation changes are shown by epidermal glands, nuclei, hook position, and parenchyma.

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EXPLANATION OF PLATE

(All figures made with the aid of camera lucida using oil immersion objectives, each scale division representing 10 microns.)

FIGURE 1. Mature appearance of epidermal gland cell; toluidine blue.

FIGURE 2. Early condition of epidermal gland cell during the period of hook formation; toluidine blue.

FIGURE 3. Post-maturation condition of the epidermal gland cell with perinuclear substance; toluidine blue.

FIGURE 4. Post-maturation condition of epidermal gland cell, thick layer of perinuclear substance; toluidine blue.

FIGURE 5. Immature oncosphere, during the early period of hook formation; epidermal gland cells shown opposite the hooks, shell capsule removed; aniline acid fuchsin.

FIGURE 6. Immature oncosphere, oblique lateral view, to show location of epidermal gland cells at bottom and the binucleate medullary center in the middle; small parenchymal nuclei included.

FIGURE 7. Mature oncosphere, just following hook completion; outer shell capsule enclosing a narrow granular membrane forming the inner capsule; Weigert's acid hematoxylin and safranine O.

FIGURE 8. Living, mature oncosphere, showing U-shaped gland area; outer shell removed, only inner capsule present; neutral red stain; gland area yellow, large cells shown red.

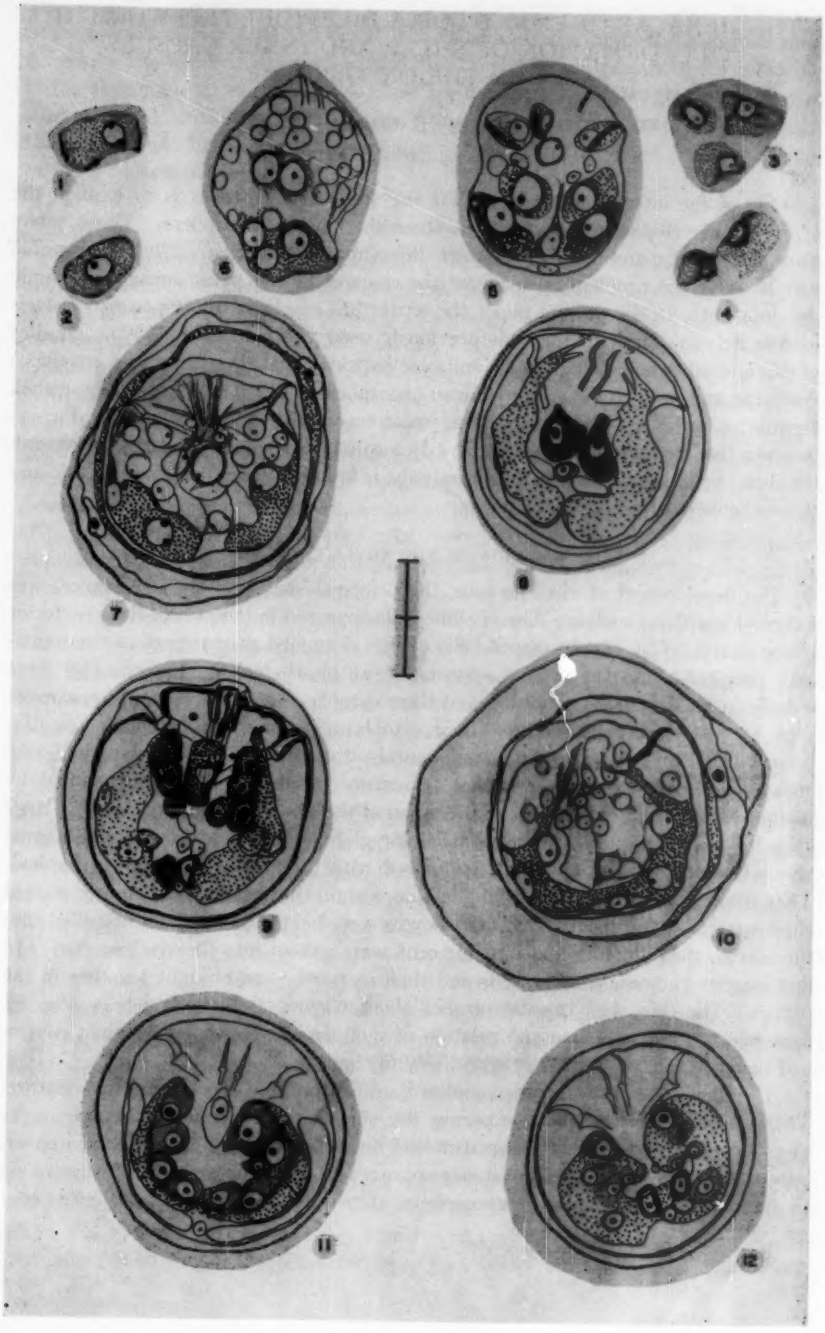
FIGURE 9. Living, mature oncosphere, outer shell capsule removed; cuticle thickened, gland area somewhat C-shaped; large dark cells colored red, gland area yellow with neutral red in vivo.

FIGURE 10. Mature oncosphere, sectioned 10 microns, with U-shaped gland area; small parenchymal nuclei in contrast with the large gland cell nuclei; terminal portions of the gland area grade into the parenchymal fibers; Weigert's acid hematoxylin and safranine O.

FIGURE 11. Post-maturation oncosphere, living specimen, outer shell capsule removed; C-shaped gland area composed of at least 8 cells at this level; neutral red, in vivo.

FIGURE 12. Post-maturation oncosphere, living specimen, 2 levels of the oncosphere superimposed; granular gland area on top, and shaded cells below; shaded part of the cell coloring red and granules yellow; neutral red, in vivo. The two very dark plastin-type cells in granular area comparable to those in figures 3 and 4.

PLATE I



THE HEXACANTH EMBRYO OF A DILEPIDID TAPEWORM. III. THE FORMATION OF SHELL AND INNER CAPSULE AROUND THE ONCOSPHERE.

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One of the interesting problems for the student of cestodes is to explain the origin and development of membranes about the mature oncosphere. These, many times, are diverse and modified by their differentiated condition. The only possible way to solve the problem is to follow the changes taking place during embryonic development. In the present paper the writer has employed the tapeworm *Dilepis undula* Schrank, and the methods previously used (Ogren, 1958, 1959). Studies of this and other similar tapeworm embryos indicated that there may be 4 envelopes enclosing and protecting cyclophyllidean oncospheres: (1) The outer shell, or shell capsule; (2) the rigid inner capsule, in some cases called the embryophore, formed between the oncosphere and the shell; (3) a colloidal or albuminous region beneath the shell; (4) in some cases, a thin membrane is found between the inner capsule and the oncosphere draped over the hooks.

FORMATION OF THE SHELL CAPSULE

The development of vitelline cells, the principal source of shell substance, was followed in *Dilepis undula*. The vitelline cells appeared in the primordium posterior to the ovary. The spindle-shaped cells of this elongated primordium in the immature proglottid had the typical appearance of plastin cells. Their nuclei were usually in an interphase condition and their cytoplasm was compact in appearance (fig. 1). As these cells enlarged, their cytoplasm became more granular (fig. 2). Somewhat older cells were larger and contained many small globules of shell substance (fig. 4). Later development in mature proglottids was accompanied by further enlargement of the cell and the union of the smaller globules into a few large globules (figs. 3, 5, 6). These intracellular globules were Schiff positive, stained blue with bromophenol blue, and bright red with the aniline acid fuchsin method. They colored blue-green with toluidine blue, a stain that usually only faintly stained shell material. During the time that oocytes were being fertilized and expelled into the uterus, shell globules and vitelline cells were passed into the vitelline duct. In this manner oocytes, vitelline cells and shell material were brought together in the uterus. The thin shell capsule present about oocytes and vitelline cells (fig. 8) appeared to form by fusion and gelation of shell droplets. As development continued the shell capsule became progressively thickened.

The shell capsule was not completed until after the oncosphere was mature. This was demonstrated by comparing the shell capsules around cleavage stages (fig. 9) with those around post-maturation oncospheres (figs. 12, 13). Moreover, only a small amount of shell substance appeared to be discharged into the uterus up to the formation of the mature oncosphere. On the other hand, in proglottids con-

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taining post-maturation oncospheres, the uterus received a great discharge of shell substance. This material formed a viscous packing in the gravid uterus. It resulted in the formation of greatly thickened shell capsules enclosing post-maturation oncospheres. The staining reactions of the thick shell capsule became more intensified in the post-maturation condition. Shell around these embryos gave the following reactions: blue with bromophenol blue, green with malachite green, purple to dull red with the allochrome periodic acid Schiff procedure, and bright red with the aniline acid fuchsin procedure.

THE INNER CAPSULE AROUND THE ONCOSPHERE

In the mature *Dilepis* egg a special protective capsule developed between the oncosphere and the shell. This membrane, absent around the morula stage, was present around the immature oncosphere accompanied by 2 or 3 large nuclei and granular material (fig. 10). One nucleus, derived from the vitelline cell, did not appear to assist in its formation. The development of the inner capsule was originally associated with certain large blastomeres, or macromeres, situated outside the morula. They enclosed the morula (fig. 11), providing an early protective layer. Later, however, these macromeres lost their visible cytoplasm and their nuclei became part of the granular capsule enclosing the developing oncosphere (fig. 12). Sometimes the nuclei were within the membranes; more frequently they were associated with their circumference. In their final condition these nuclei became wrinkled, elongate and difficult to identify. The real part played by these macromeres has not been fully ascertained.

The granules of the inner capsule (fig. 12) were spheres, ovals, or short rods. They stained black with hematoxylin in the metachrome procedure, pink with aniline acid fuchsin, and red with the allochrome periodic acid Schiff procedure. They represented dispersed particles from the macromere cytoplasm, and may contain mitochondria. In subsequent development the granules disappeared and the inner capsule appeared as a nearly homogeneous membrane (fig. 13).

The inner capsule became thicker and more evident during development. This was demonstrated by comparing membranes around early and late oncospheres. In their early period of hook formation, oncospheres were enclosed by shell and a single thin membrane associated with the macromere nuclei (fig. 10). On the other hand, in their post-maturation condition, the oncospheres were enclosed by the shell and a thick, transparent, slightly lamellated inner capsule (fig. 13). Usually an additional delicate membrane was draped over the hooks.

Chemical changes during development to a post-maturation condition were indicated by a decrease in affinity of the inner capsule for certain counterstains. For example, the inner capsules around mature oncospheres colored intensely with safranine O, pyronin, toluidine blue and the Schiff procedure; whereas, inner capsules around post-maturation oncospheres were only faintly stained or not at all.

THE REGION BENEATH THE SHELL

A definite physiologically important region developed beneath the shell. It was not pronounced, however, until the hooks had begun to form (fig. 10). At this time there was an abundance of granules beneath the shell; large macromere nuclei were also identifiable. When hook formation was completed, the inner capsule was

present, the outer shell had thickened (fig. 13), and a broad region of viscous material was present beneath the shell. This material had a gel consistency and had metachromatic properties, colored red with pyronin and positive red with Schiff procedure for glycogen. This subshell zone was the broadest and most metachromatic in the post-maturation condition of the embryo.

The 3 envelopes about the oncosphere, the shell capsule and colloidal subshell region, and the inner capsule showed different staining reactions. These differences were manifested in such a way that the outer shell capsule stained a more brilliant color in the post-maturation egg. For example, with bromophenol blue, the shell was blue, the inner capsule was greenish, and the sub-shell region practically colorless. When stained with aniline acid fuchsin, the shell was bright red, inner capsule only faintly pink, the sub-shell region contained scattered pink granules and 1 or 2 pink nuclei. With pyronin, shell and inner capsule were unstained, the sub-shell region was stained red along with the epidermal glands.

DISCUSSION

Early investigators describing tapeworm eggs fully realized the important part played by the extra-embryonic membranes and the shell. However, they did not always describe the manner of their origin. The excellent work of Smyth (1956) has called special attention to the cytological basis of egg shell formation. There are 4 aspects to this general problem: (1) The production of egg shell substance in the vitelline cell cytoplasm; (2) the construction of the shell capsule from droplets of shell material during cleavage and oncosphere development by a process perhaps common to all tapeworms; (3) the kind of uterus in which development occurs; and (4) the condition or age of the embryo when the shell capsule is actually completed. In Pseudophyllidea having a tubular trematode-like uterus, it was shown by Smyth (1956) that a thick capsule was constructed in the proximal part of the uterus in a manner similar to trematodes. Subsequent changes involved a quinone tanning in the more distal parts of the uterus. In certain Cyclophyllidea, on the other hand, in the young uterus construction begins around cleavage stages. The shell is not fully completed until a gravid uterus has been attained and the oncospheres are in the post-maturation condition. This process accounts for statements in the literature describing how the uterine contents break up into separate capsules. This phenomenon is actually the completion of the shell capsule and the resulting isolation of the hexacanth embryos from each other.

Since properties of these protective membranes permit survival in the external environment and successful entrance into the host, it is important to investigate their chemical nature. Literature about the outer shell capsule has been summarized by Smyth (1956). The outer shell capsule in pseudophyllideans consists of the protein sclerotin. Selective coloration with malachite green and blue coloration with bromophenol blue are considered positive evidences for sclerotin. In *Dilepis undula*, a cyclophyllidean with hymenolepidid-type oncospheres containing epidermal glands, the post-maturation egg shell was positive for sclerotin with both reactions.

The chemical nature of the inner capsule is not well known. Its stain reactions change during development to become more refractory to counterstains. It did not react for sclerotin as did the shell, but gave reactions similar to cuticle, muscle, and

proteins such as collagen.

The material beneath the shell appears to have definite metachromatic properties and may be some viscous glycoprotein that reduces permeability. However, the source of this material is unknown. It is suggested that it arises from the developing oncosphere and the dispersed cytoplasmic products of the macromeres.

Glycogen and phospholipids have been identified in the region between the oncosphere and the shell in eggs of *Hymenolepis diminuta* and *Raillietina cesticillus* by Hedrick and Daugherty (1957) and Hedrick (1958). Schiff-positive and metachromatic substances were demonstrated in the region between the shell and oncosphere in eggs of *Oochoristica symmetrica* by the writer (Ogren, 1957). Considerable histochemical work remains to be done. It is evident that this extraembryonic region is very important to the survival of the embryo and that because of the disintegration of cells in this region various cytoplasmic products will be found here.

In studying the physiology of membranes about the hexacanth embryo, one must consider their manner of development and the type of oncosphere they enclose. A true comparative study will consider the hymenolepidid and the taeniid types, for example, as suitably different for comparisons. However, *Dilepis, Hymenolepis,* and *Raillietina cesticillus* are all of the same basic type.

Experimental studies are now needed on the properties that give these membranes adaptive significance and promote survival of the infective hexacanth embryo. Some of these are: (1) chemical composition and digestibility by various enzymes; (2) colloidal properties, viscosity, gelation, rigidity, color, elasticity; (3) permeability to entrance or exit of various substances, e.g., water, salts, organic compounds, and oxygen.

The hatching of tapeworm eggs has been observed in several cases and may be defined as the release of the oncosphere from the enclosing membranes. In the excellent studies of Silverman (1954) on *Taenia* it was shown that in studying hatching one is really studying the chemical properties of the shell and inner capsule. In the present study the writer has realized that the developmental condition of the membranes is a factor that must be known in future experimental work. The immature soft membranes rupture readily and are more permeable than the completed post-mature ones. Artificial hatching is thus more easily accomplished by mature than by post-mature oncospheres. The post-maturation condition, however, is more resistant to desiccation and injurious environmental conditions.

SUMMARY

The mature oncosphere was enclosed by 3 basic layers or egg envelopes. 1. The external shell capsule was formed from vitelline cell products and was not completed until late in development in the most gravid proglottids. This shell gave positive reactions for sclerotin. 2. A sub-shell colloidal envelope developed after the oncosphere became mature. This layer had metachromatic properties and formed a further barrier to diffusion. 3. An inner capsule appeared around the early oncosphere. It developed in association with certain macromeres and secretory products, and became a thick, rigid membrane. Changes in chemical nature of these envelopes was shown by changes in their affinity for certain counterstains. Schiff-positive substances increased during development in the region between the shell and oncosphere.

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Explanation of Plate I

All figures were made with the aid of a camera lucida attachment using oil immersion objectives. Each scale division equals 10 microns. Abbreviations: BMC, binucleate medullary center; COL, colloidal layer beneath the shell; EG, epidermal gland cell; IC, inner capsule; M, macromere; MN, macromere nucleus; ON, oncoblasts; PB, polar bodies; PN, parenchymal nuclei; SC, shell capsule; SG, shell globules; SM, vitelline material in macromere; VIT, vitelline cell; VN, vitelline cell nucleus.

Figure 1. Early vitelline cell in immature gland; toluidine blue.

Figure 2. Vitelline cell, immature, from peripheral part of vitelline gland; toluidine blue.

Figure 3. Vitelline cell, immature, from central part of the vitelline gland; toluidine blue.

Figure 4. Young vitelline cell; allochrome Schiff, globules pink.

Figure 5. Older vitelline cell; allochrome Schiff, globules pink.

Figure 6. Oldest vitelline cell, allochrome Schiff, globules red.

Figure 7. Vitelline cell associated with an oocyte in the uterus; allochrome Schiff.

Figure 8. Group of 3 oocytes in the uterus enclosed by shell material; allochrome Schiff, shell material red.

Figure 9. Early cleavage stage; van Gieson's connective tissue stain.

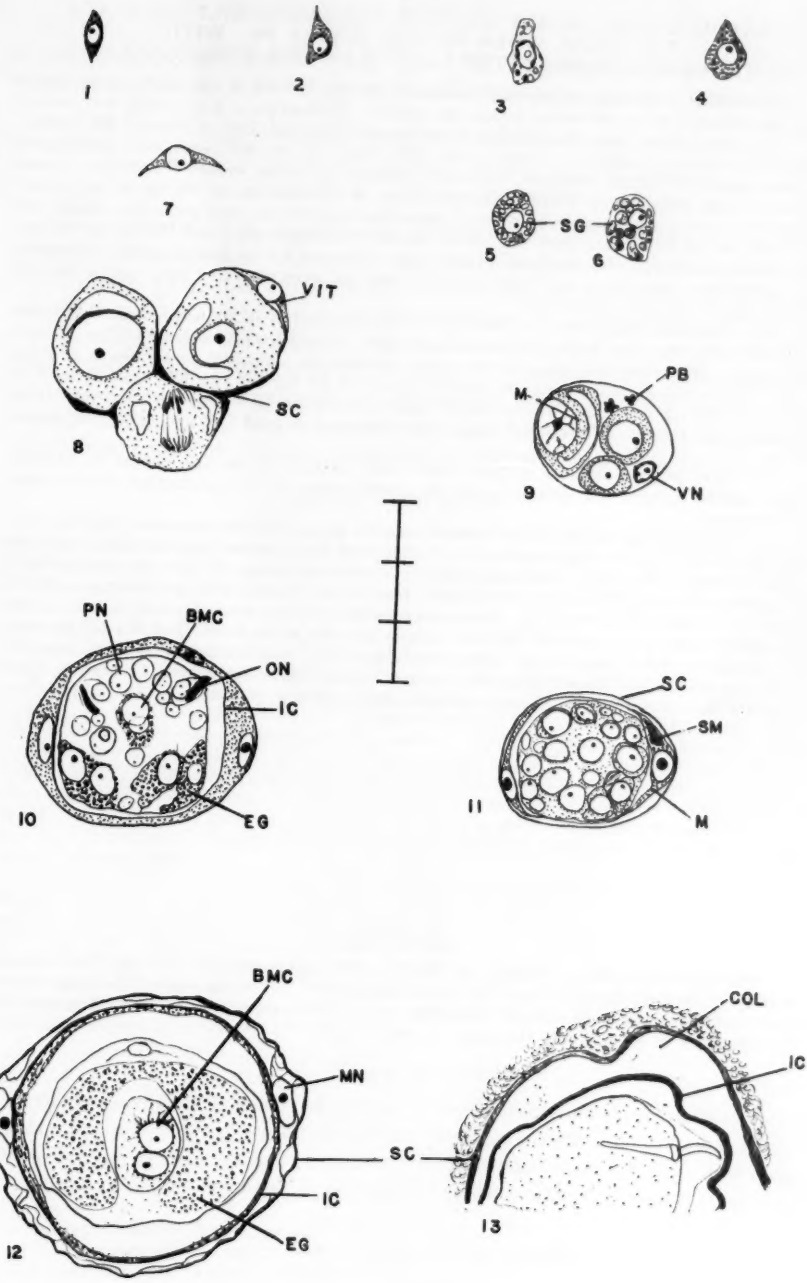
Figure 10. Oncosphere, early period of hook formation; metachrome procedure with Weigert's hematoxylin and safranine O.

Figure 11. Embryo, late morula stage; methyl green-pyronin.

Figure 12. Mature oncosphere enclosed by shell and inner capsule; very little free shell substance in the uterus; metachrome stain, inner capsule pink, granules black.

Figure 13. Post maturation condition of the membranes; oncosphere, only partly shown here, enclosed by a thickened shell and thick inner capsule; toluidine blue stain, the colloidal sub-shell material purple, (A large amount of shell material packed into the uterus of these terminal proglottids.)

PLATE I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RESEARCH NOTES

ANTHELMINTIC TRIALS AGAINST *PARASPIDODERA UNCINATA* THE CECAL WORM OF THE GUINEA PIG, WITH OBSERVATIONS ON THE LENGTH OF THE NEMATODES.

Paraspidodera uncinata, a heterakid nematode parasite located in the cecum of the guinea pig, was detected in the laboratory guinea pig colony. Elimination of the parasite was desired.

It has been shown that phenothiazine (Guthrie and Harwood, 1942, J. Parasit. **28** (supp.): 24) and piperazine hexahydrate (Edgar et al, 1957, Poul. Sci. **36**: 495) have some anthelmintic activity against a related nematode, *Heterakis gallinae*, the cecal worm of chickens. Consequently, single oral doses of phenothiazine were given at a dosage rate of 440 mg per kg of body weight to 3 guinea pigs, single oral doses of piperazine hexahydrate were given at a dosage rate of 75 mg per kg to 4 guinea pigs, and 150 mg per kg to 2 guinea pigs, and 150 mg per kg was given for 3 consecutive days to 2 other guinea pigs. None of the 11 treated animals eliminated any worms with their feces, and from 6 to 23 *P. uncinata* were recovered from each guinea pig at necropsy 5 days after treatment.

Since the above drugs were ineffective in these trials, and as it has been shown that ronnel (Dow ET-57) has some anthelmintic activity in cattle (Herlich and Johnson, 1957, J. Parasit. **45** (supp.): 19), single oral doses of this organic phosphorus compound were given at a dosage rate of 100 mg per kg to 2 guinea pigs and to 2 others at 200 mg per kg. One male *P. uncinata* was expelled by a guinea pig treated at the higher dosage but 15 worms were recovered from the animal at necropsy. The other 3 guinea pigs eliminated none of 5, 9, and 15 worms, respectively.

The three drugs as utilized in these trials were obviously ineffective against *P. uncinata*. These results indicate that this host-parasite system may not be satisfactory for screening anthelmintics.

An average of 14.2 worms was recovered from the 15 experimental animals. This compares very closely with the average number (13.39) reported in 61 guinea pigs by Porter and Otto (1934, J. Parasit. **20**: 323). The nematodes recovered were compared with the descriptions of *P. uncinata* given by Travassos (1914, Mem. Inst. Osw. Cruz **6**: 137) and Pereira and Vaz (1933, Rev. Biol. e Hyg. **4**: 52). They corresponded in every particular except total length. Maximum length in mm recorded by those authors was 19 for the female and 17.4 for the male. The specimens of the present study ranged from 23.6 to 27.5 long for the females and from 17.5 to 21.6 for the males.—HARRY HERLICH, *Regional Animal Disease Research Laboratory, Agricultural Research Service, U. S. Dept. of Agriculture, Auburn, Alabama.*

ERRATUM

Journal of Parasitology, Volume 45, No. 2, p. 190, last paragraph, line 15. The sentence starting "The tough, digestion-resistant hard-walled cysts . . . ." should read: "The tough-walled cysts containing digestion-resistant organisms . . . ."—HERBERT E. KAUFMAN, MARJORIE L. MELTON, JACK S. REMINGTON, AND LEON JACOBS.

AUTORADIOGRAPHIC STUDY OF THE SYNTHESIS OF RIBONUCLEIC ACID IN CYSTICERCOIDS OF *HYMENOLEPIS DIMINUTA**

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Previous studies of the development and metabolism of hymenolepidid cestodes have included experiments dealing with the effect of temperature on development and differentiation (Voge and Turner, 1956; Voge and Heyneman, 1958), and observations on the distribution of various substances within the cysticercoid stages of these organisms (glycogen distribution, Heyneman and Voge, 1957; fat distribution, Voge, 1960). The present report describes the localization of ribonucleic acid synthesis within the cysticercoids of *Hymenolepis diminuta*. Since RNA is apparently intimately involved in protein synthesis, information about such activity should extend our understanding of growth and differentiation of this cestode.

MATERIALS AND METHODS

Animal strains and infection procedures were as described previously by Voge and Heyneman (1957). Cysticercoids which had developed at 30° C were dissected from beetles in mammalian physiological saline. They were then washed in several changes of saline, and incubated for 3 or for 5 hours at 37° C in the saline medium containing 2μc/ml of C-14 adenine. Following this treatment, the larvae were freed of external radioactivity by a series of washings in cold physiological saline. They were fixed in cold Bouin's fixative for approximately 30 minutes and extracted in cold 70 percent alcohol for several hours in order to remove any unincorporated RNA precursor. They were then passed through a graded series of chilled alcohols to xylene, embedded in paraffin or in gelatin, sectioned at 10 microns, and mounted on slides in the usual manner. Light staining with eosin-y in 95 percent alcohol, prior to embedding, greatly facilitated examination of slides for the presence of sectioned material. For control purposes, 2 slides were incubated in ribonuclease (0.1 mg per ml) adjusted to pH 7.0 by phosphate buffer for 20 minutes at 22° C. Autoradiography of these slides showed that all incorporated radioactivity was removed by this treatment. As a further control, 1 slide was kept in distilled water buffered to pH 7.0 with the same buffer as above at 22° C for 20 minutes. Radioactivity was retained in spite of this treatment. Radioactivity of fixative and alcohols was checked after use and was proved to be negligible.

The incorporation of C-14 adenine was detected by standard autoradiography. To prevent chemical exposure of the autoradiographic emulsion, the slides from the paraffin embedded material were first dipped into 1 percent celloidin in ether and absolute alcohol, and allowed to drain and dry. This procedure was repeated. The slides were then covered with Kodak Autoradiographic Film AR-10, and the film exposed for 3 weeks. The autoradiographs were developed for 6 minutes in Kodak D-11 at 24° C. Using a Whipple disc, radioactivity was quantitated by silver grain counts (table I) on representative 309 μ^2 areas (see fig. 2) on a number of sections from several organisms.

RESULTS AND DISCUSSION

Incorporated C-14 adenine has proven to be a reliable indicator for RNA synthesis. In a proliferating population of cells, some C-14 adenine is incorporated into DNA, but the amount is small relative to that incorporated into RNA. The autoradiograph of the *Hymenolepis* cysticercoid in figure 1 may reflect slight incorpora-

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tion of C-14 adenine into DNA of multiplying cells, but it is quite reasonable to assume that most of the radioactivity represents the synthesis of RNA. It is apparent from figure 1 that there are marked differences in rate of RNA synthesis from one tissue or area to another. The synthetic activity is most concentrated in the peripheral layer of tissue and at the body-tail junction. Synthetic activity is less in the scolex and least in the area between the cysticercoid cavity and the peripheral layer. The data in table I represent grain counts of autoradiographs over 1 or 2 sections from 10 or more different organisms.

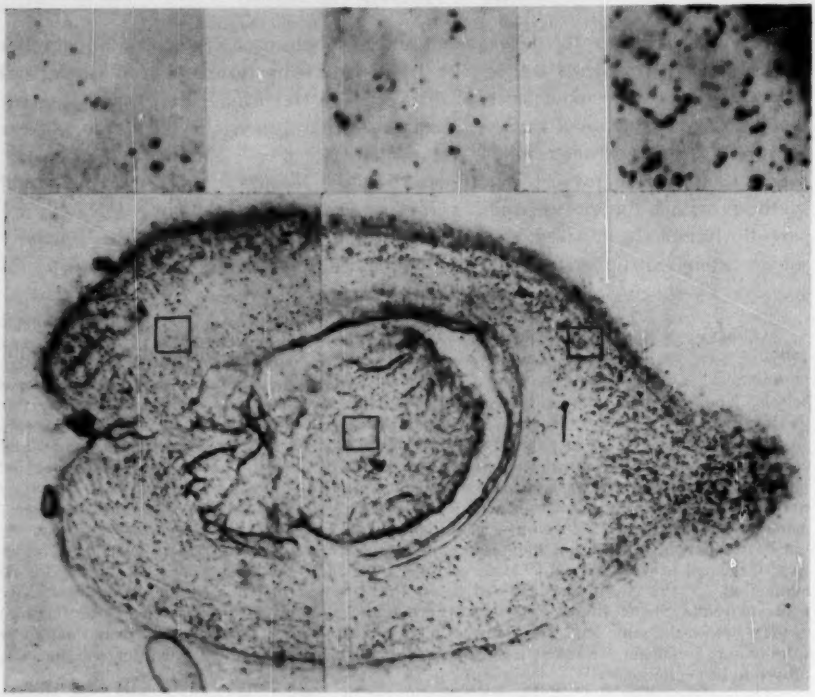

FIGURE 1. Lower photograph an autoradiograph of *Hymenolepis diminuta* cysticercoid (section) labelled with C-14 adenine, showing distribution of silver grains. Incorporated radioactivity is most intense in the periphery and at the body-tail junction. Upper figures are enlargements of three representative areas (see squares) photographed at silver grain level.

The specific role of RNA in cellular metabolism is not definitely established. There is good evidence, however, that RNA is essential to protein synthesis and that its presence reflects a high level of such synthesis in the cytoplasm. The concomitant occurrence of abundant RNA and protein synthesis is characteristic of certain specialized, non-multiplying cells producing extra-cellular protein, and all cells which are in a state of steady proliferation.

The high rate of RNA synthesis in the body-tail junction is probably connected with cellular proliferation which must occur since tail growth continues in the fully developed cysticercoid (Voge and Heyneman, 1957, p. 553). Conversely, the relatively low rate of synthesis in the scolex supports the observation that the scolex is fully differentiated at this stage and apparently has ceased to grow. It is also evident

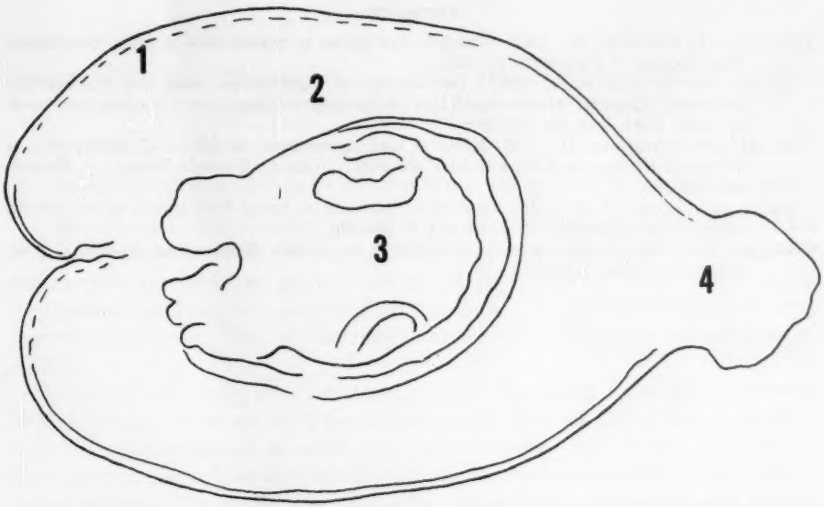

FIGURE 2. Diagram of section in figure 1, showing four areas in which radioactivity was quantitated by silver grain counts: 1, outer membrane and peripheral layer of tissue; 2, tissue between peripheral layer and cavity enclosing scolex; 3, scolex; 4, body-tail junction.

that RNA synthesis, and therefore all proliferation, is distinctly limited in the tissues immediately surrounding the cysticercoid cavity (fig. 1). We have no satisfactory explanation for the elevated rate of synthesis in the peripheral layer which is histologically a distinct, well-defined tissue (Voge and Heyneman, 1957, pp. 552, 571). The function of these cells is unfortunately unknown. Recent studies on cysticercoid histology and cytology (Voge, to be published) show that the peripheral tissue layer consists of flask-shaped cells which, after withdrawal of the cysticercoid scolex, give rise to numerous hair-like processes. This activity is perhaps related to the relatively high rate of RNA synthesis.

TABLE I. *RNA synthesis determined by silver grain counts on four areas of sectioned cysticercoids of* Hymenolepis diminuta.

	Outer membrane and peripheral layer	Body-tail junction	Scolex	Area between peripheral layer and cysticercoid cavity
No. sections counted	10	10	13	14
Average no. of grains/square	69	51	31	27
Standard error	±3	±5	±3	±1

SUMMARY

The intensity of ribonucleic acid synthesis was investigated in cysticercoids of *Hymenolepis diminuta* by means of the C-14 adenine and autoradiographic procedure. The highest rate of RNA synthesis occurs in the body-tail junction and in the peripheral tissue layer of the cysticercoid. Relatively little synthesis takes place in the scolex and in the area immediately adjacent to the cysticercoid cavity. Possible reasons for some of these differences are discussed.

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TEMPERATURE STRESS AND DEVELOPMENT OF *HYMENOLEPIS DIMINUTA* IN *TRIBOLIUM CONFUSUM* ON DIFFERENT DIETS*

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The existence of a sensitive period in the development of cysticercoids of the cestode *Hymenolepis diminuta* has been demonstrated (Voge, 1959) by exposing these organisms to high temperature stress. Exposure to 38.5° C at days 3 to 5 of development produces two general effects: failure of scolex withdrawal in a large number of larvae, and lack of infectivity to rats by morphologically normal cysticercoids.

In the present study, an attempt was made to determine if the results obtained by high temperature stress could be modified by changing the diet of the intermediate host *Tribolium confusum* in which the cysticercoids develop. It was thought that host nutrition might have considerable influence on the development of the parasite in the presence of stress because of possible competition between host and parasite for essential foods.

The dependence of adult *Hymenolepis* on certain carbohydrates has been demonstrated by Read and Rothman (1957) and by Read et al (1958). Since the food of *T. confusum* consists principally of carbohydrate, it was assumed, in the presence of stress, that the host might compete more successfully and that a scarcity of energy-yielding materials might prevent normal parasite development.

Accordingly, beetles were fed pure sugars, either dry or in aqueous solution, during the total period of parasite development. Previous studies (unpublished) have shown that adult beetles can live for one month or longer on a diet of certain pure sugars, and that normal and infective cysticercoids will develop in these beetles at 30° C. Subsequently, it was found that not only adult but also larval *Tribolium confusum* can serve as effective intermediate hosts for *H. diminuta,* whether on the usual flour diet or on pure sugars. Therefore, some of the results obtained with *Tribolium* larvae are also included here.

Appreciation is expressed to Mrs. Gerta Barlow and to Miss Mary Mullee for technical assistance and helpful suggestions.

MATERIALS AND METHODS

Animals and infection procedures were as previously described by Voge (1959). Care was taken that beetles were always starved for exactly 1 week prior to infection. Experimental groups were fed on eggs for 6 hours and were then transferred to containers with pure sugars. (The source for maltose, sucrose and fructose was Difco Laboratories, Detroit, Michigan; glucose was obtained from General Chemical Division, New York; galactose, raffinose and arabinose from Pfanstiehl Laboratories Inc., Waukegan, Illinois.) These were fed dry or in the form of a saturated aqueous solution. The solutions were pipetted on a small piece of cotton lying on a glass coverslip on top of the filter paper lining the bottom of the container. Care was taken to keep the cotton moist at all times. Beetles were exposed to 38.5° C on days 3 to 5 of cysticercoid development. At all other times they were kept at 30° C. Experimental diets were fed for

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the total period of cysticercoid development which is 8 days. For each experiment controls were maintained on enriched flour. When flour and water were provided, the water was given on cotton as described for the sugar solutions.

In most experiments humidity was not controlled. Measurements taken in the 30° C incubator for one month showed a range in relative humidity of 14–28 percent. In other experiments, the beetles were placed in special desiccators containing phosphorous pentoxide which reduced relative humidity to 0 percent, or water which raised the relative humidity to 76 percent or more (see below). A hygrometer placed in the desiccators for the duration of the experiment permitted daily humidity determinations without opening the jars.

Eight days after eggs had been fed to beetles or beetle larvae the hosts were dissected in normal saline solution, the numbers of withdrawn and non-withdrawn cysticercoids recorded and some of the apparently normal cysticercoids fed to rats to determine infectivity. Rats were examined 2 or 3 weeks later for the presence of adult worms. These were then fixed in Bouin's fluid, stained with a dilute solution of Ehrlich's hematoxylin or with Best's carmine, and examined for morphological abnormalities.

All data presented here are based on many experiments with moderate infections so as to rule out, as far as possible, the effects of crowding. Rate of cysticercoid development in beetles on sugar diets was checked and found to be the same as in beetles fed on flour. Mortality of beetles on the different carbohydrate diets was recorded and will be discussed. Certain diets such as casein (with or without vitamins) resulted in death of all beetles within a few days and therefore could not be used.

RESULTS

Effect of Host Diet on Cysticercoid Morphology

The effects of different host diets on withdrawal of the scolex of the developing cysticercoid are shown in table I. The data represent totals of several to many experiments. Environmental humidity was not controlled, and varied as stated in the section on methods. As can be seen from comparison of results obtained with flour controls with those from various sugar diets, the ratio between non-withdrawn and withdrawn larvae changes markedly depending on the diet of the beetle.

TABLE I.

THE EFFECT OF DIFFERENT DIETS OF TRIBOLIUM CONFUSUM ON THE DEVELOPMENT OF HYMENOLEPIS DIMINUTA IN THE PRESENCE OF TEMPERATURE STRESS

FLOUR ONLY (CONTROL)		FLOUR + WATER		DRY GLUCOSE		SAT.* GLUCOSE	
NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN
793 (68%)	380 (32%)	119 (53%)	104 (47%)	108 (43%)	138 (57%)	79 (28%)	199 (72%)
DRY FRUCTOSE		SAT. FRUCTOSE		DRY GALACTOSE		SAT. GALACTOSE	
NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN
45 (37%)	77 (63%)	56 (24%)	177 (76%)	6 (15%)	35 (85%)	25 (14%)	152 (86%)
DRY MALTOSE		SAT. MALTOSE		DRY SUCROSE		SAT. SUCROSE	
NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN
444 (59%)	315 (41%)	210 (33%)	425 (67%)	135 (51%)	130 (49%)	114 (26%)	325 (74%)
DRY ARABINOSE		SAT. ARABINOSE		DRY RAFFINOSE		SAT. RAFFINOSE	
NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN	NOT WITHDRAWN	WITHDRAWN
0 NOT TOLERATED BY HOST	0	7 (12%)	52 (88%)	103 (38%)	166 (62%)	47 (25%)	140 (75%)

* SATURATED SOLUTION

In general, three different types of reactions are observed. The control group, fed on flour only, shows a much larger number of non-withdrawn than withdrawn larvae. The second group, in which approximately one-half of the larvae have failed to withdraw, results when drinking water is supplied in addition to flour, or when beetles are fed on certain dry pure sugars such as glucose and sucrose. Beetles fed on certain dry sugars, and on saturated sugar solutions make up the third group in which the proportion of withdrawn to non-withdrawn cysticercoids is 2 to 1. This is a reversal of the ratio obtained from the control group (see table I, flour only, contrasted with saturated maltose). A ratio of 3 to 1 withdrawn to non-withdrawn is obtained with saturated sucrose and raffinose; but since similar ratios were obtained in isolated experiments with maltose, it would be premature to state that these 2 sugars have a more pronounced effect than maltose.

Data in table I are obtained by using adult *Tribolium*. Experiments were also performed with *Tribolium* larvae which were treated in the same manner as the adults. When fed on dry flour and exposed to 38.5° C on days 3–5 of cysticercoid development, 159 (72 percent) of the cysticercoids fail to withdraw the scolex, while 61 (28 percent) withdraw normally. When *Tribolium* larvae are fed on the pure sugars, the ratios of withdrawn to non-withdrawn cysticercoids are similar to those shown in table I.

To determine the effect, if any, of low or high environmental humidity using adult beetles or larvae, humidity was either reduced or raised, and maintained at a certain level for the duration of experiments. Results of preliminary studies using *Tribolium* adults and larvae show that the numbers of withdrawn and non-withdrawn cysticercoids obtained at 0 percent or at 76–89 percent relative humidity are very similar. When adult beetles were fed on flour and kept at 0 percent humidity, 59 percent of the cysticercoids are non-withdrawn, while in beetle larvae 69 percent were non-withdrawn. At 76–89 percent relative humidity, 67 percent of the cysticercoids are non-withdrawn in beetles on a flour diet. When fed on saturated maltose, only 45 percent from adult beetles and only 28 percent of the cysticercoids from beetle larvae are non-withdrawn. However, it is difficult to perform equivalent experiments because under conditions of high humidity the dry flour absorbs much moisture and becomes stiff, thus perhaps interfering with the feeding process, while with low humidity the liquid either evaporates very quickly or the humidity cannot be maintained. This is why experiments with saturated maltose were not feasible in the presence of low humidity.

Effect of Host Diet on Infectivity of Cysticercoids to Rats

Because of the pronounced morphological effects on cysticercoids from beetles on sugar diets, infectivity of these cysticercoids to rats was tested and compared with the flour controls. Only fully withdrawn and apparently normal cysticercoids from adult beetles were used. Results are shown in table II, which lists data for individual experiments. It is apparent that rate of infectivity is considerably higher in cysticercoids from beetles fed on sugars than from those fed on flour only. The totals are as follows: 72 cysticercoids from flour-fed beetles yield only 3 adult worms (4 percent infectivity); 183 cysticercoids from beetles on sugars or flour and water yield 33 adult worms (18 percent infectivity). It should be stressed that infectivity of normal cysticercoids is rarely 100 percent if more than 2 are fed per rat. Of 150 normal cysticercoids developed at 30° C and fed to 30 rats (5 per

rat) only 112 adult worms were recovered (74.6 percent infectivity). The reasons for these losses remain to be determined. When only 1 or 2 cysticercoids are fed, percent infectivity is usually higher.

Examination of stained adult worms obtained from experiments listed in table II shows that they could not be differentiated from normal adults. All contained embryonated eggs. Volume determinations were not made.

TABLE II. *Infectivity of cysticercoids developed in* Tribolium confusum *on different diets, in presence of temperature stress.*

Nutrient	No. rats infected	Cysticercoids fed/rat	Worms recovered
Flour only	2	6	0
	2	5	0
	4	3	0
	2	3	0
	2	6	1
	1	6	0
	2	2	0
	1	4	0
	1	6	2
Flour + Water	2	2 and 3	1
	1	2	0
Sat. glucose	2	5	0
	2	6	4 (2 rats)
Dry fructose	2	6	4 (2 rats)
Dry galactose	2	6	1
Dry maltose	2	5	0
Sat. maltose	1	6	0
	6	3	4 (one rat)
	7	3	2 (" ")
	2	4	0
	2	5	4 (2 rats)
Dry sucrose	1	3	0
	2	5	1
Sat. sucrose	2	6	1
	2	5	2 (one rat)
Dry raffinose	2	6	8 (2 rats)
Sat. raffinose	2	6	1

Tolerance of Adult Tribolum confusum *to the Different Diets With and Without Temperature Stress*

Observations on approximately 800 beetles on different diets show that, with or without temperature stress, certain diets are well tolerated and others poorly or not at all. Beetles kept on dry flour or on any of the lower sugars, dry or in solution, show comparable, low mortality (10 percent or less). Raffinose was equally well tolerated. High mortality occurred with galactose and with saturated arabinose. All beetles died when fed on dry arabinose.

DISCUSSION

From the results reported it is evident that the effects of temperature stress on the developing parasite may vary considerably, depending on the diet of the host. The present situation is complex, because of the intimate association between 2 organisms, the host and the parasite. Unless the host provides a suitable environment, development of the parasite will not proceed normally. Under conditions of stress, the fully grown, and presumably more resistant host may utilize certain substances more efficiently and cope with environmental stresses to the detriment of the developing parasite. Conversely, if the host environment is modified, deleterious effects of stress on the parasite can be reduced or perhaps even eliminated depending on the intensity or the duration of stress. Studies are in progress to determine the

maximal duration of temperature stress, the effects of which can be compensated for by diet.

Two major factors appeared to be responsible for the reduction of high temperature effects on the cysticercoids: availability of fluid, and an increase in dietary carbohydrate. A host diet of pure sugar was beneficial to the developing cysticercoid. This effect was enhanced when the sugars were fed in solution. While it is probably true that food intake of the beetles is increased in the presence of a liquid diet, which might be more palatable, and that increased intake of carbohydrate is important in producing the effects described, it was also shown that the addition of water to the flour is at least to some extent effective. It is therefore likely that dehydration of the host under stress, while seemingly not critical for the host, may affect its metabolism in such a way as to impair development of the cysticercoid. Experiments with low and with high environmental humidity suggest that for *Tribolium* environmental humidity is of relatively little importance if water is provided with food.

When in their normal environment, insects are generally well protected from water loss through the body surface. Loss of water from the tracheal system is regulated by means of the spiracles (Edwards, 1953). Water loss by evaporation from the cuticle is controlled by a lipoid layer in the epicuticle. However, at high temperatures this layer may loosen, with the result that evaporation is increased. Richards (1953) discusses water loss through the cuticle and in a chart shows the rate of evaporation in different species of insects as a function of environmental temperature. In *Tenebrio* larvae at 40° C, water loss is shown to be less than 1 mg/cm²/hr. However, water loss at any given temperature varies considerably from one insect species to the next, and comparable data for *Tribolium* are not available. From observations on *Tribolium confusum* it seems that water loss through the cuticle either is not very great, or can be compensated for if liquids are provided in the diet. For cysticercoid development, environmental humidity outside of the host is not an important factor, because development under stress is similar whether humidity is high or low.

Utilization of different carbohydrates by *Tribolium* larvae has been studied by Bernard and Lemonde (1949), who showed that sucrose was the most effective carbohydrate source, but that glucose, maltose, raffinose, and other sugars not used in the present study were nearly as suitable. Arabinose and galactose, however, had no nutritional value and pupation of larvae was prevented. These observations are in general accord with results obtained here with adult *Tribolium*. The adult beetles are able to utilize all the sugars tested except arabinose and galactose, which are not satisfactory. In spite of high mortality, it was possible to maintain a small number of beetles on galactose, and on arabinose in solution, for the duration of the experiment. That this represents utilization rather than just survival of starvation, is indicated by experiments in which beetles were exposed to high temperatures, but were given water only without any food. This resulted in death of all beetles before termination of the experiment. Further indication of the utilization of these sugars is seen in the effect produced on cysticercoid development (table I).

While results with the sugar diets strongly suggest the superiority of most of these sugars over wheat flour for short-time experiments and conditions of stress, there is no satisfactory explanation for the precise mechanism involved in the "protective" effect on the cysticercoids. Perhaps, the simple assumption that increased

energy requirement of both host and parasite in the presence of temperature stress is satisfied by these experimental diets is sufficient. It is not yet known, however, whether larger amounts of carbohydrate are actually utilized by the cysticercoids under stress. Preliminary experiments have shown that glucose uptake of these cysticercoids is much increased over uptake of controls at equivalent stages of development. Yet nothing is known about glucose utilization.

In all experiments discussed here, beetles were fed the experimental diets for the total period of cysticercoid development. If this were done only for the duration of the stress period, differences might be less marked. Studies to answer this question are in progress.

Finally, it is clearly desirable to undertake studies on other metazoan invertebrates, symbiotic and free-living, to determine the effect of different diets, particularly carbohydrates, in the presence of stress during sensitive periods of development. Until different organisms are studied, it will not be known whether diets rich in carbohydrates can modify effects of high temperature stress in organisms with varied nutritional habits or only in those species for which a high carbohydrate diet is a normal requirement.

SUMMARY

Studies to determine the influence of host diet on development of cysticercoids of *Hymenolepis diminuta* in *Tribolium confusum* in the presence of temperature stress show the following. Deleterious effects of high temperature stress during the sensitive period of cysticercoid development can be markedly reduced by feeding *Tribolium* adults or larvae pure sugars instead of whole wheat flour. Addition of water to a flour diet also results in modification of results obtained with flour alone. The most marked effects are obtained with diets consisting of saturated aqueous solutions of glucose, fructose, galactose, maltose, sucrose, arabinose or raffinose, which result in only one-third or less morphologically abnormal cysticercoids as compared to two-thirds or more abnormals when the host is fed on flour. Furthermore, percent infectivity of cysticercoids to rats shows a marked increase when beetles are fed on sugars, dry or in solution. The importance of environmental humidity and host tolerance of the experimental diets in relation to temperature stress are discussed.

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HOST-FLEA RELATIONSHIPS IN THE GREAT SALT LAKE DESERT*

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Information regarding fleas of the Great Salt Lake Desert has been compiled primarily by independent collectors who, for the most part, were unable to concentrate their collecting efforts in the locality for long periods of time. Stanford (1944) lists several species taken from rodents. Allred (1952) and Beck (1955) have published records of potential and known vectors of sylvatic plague. Others have contributed either directly or indirectly to the understanding of certain genera in this or proximate areas having similar habitat types. The combined information, though fragmentary, forms a worthwhile contribution to the knowledge of the fleas of Utah.

An opportunity to initiate a more detailed study was presented in August, 1951, when the Army Chemical Corps organized the Wildlife Research Branch of Medical Investigation Division at Dugway Proving Ground, Utah. The project, later placed under contract to the University of Utah, continued from December 1, 1952, to the present. Continuous and rather comprehensive collections of fleas and other ectoparasites were made in all seasons of the year during both administrations. Throughout the investigations the host-flea associations were recorded, a few heretofore unrecorded associations were discovered in the area, and several new locality records were established. The list of host-flea associations was prepared solely from these collection records.

The bulk of the material in this study was collected in Dugway Valley, situated at the southern end of the Great Salt Lake Desert, near the central part of Tooele County, Utah. Other collection areas include most of the western portion of Tooele and Juab Counties and northwestern Millard County, and the Stansbury and Onaqui mountains in eastern Tooele County.

Specimens collected by the authors were supplemented by collections made by several members of the staff. Others cooperated by furnishing specimens while conducting related studies such as endemic disease surveys, mammal and bird taxonomy, life history, specific seasonal ectoparasite surveys and general collecting. Fleas from each of the above sources, after having been brushed from hosts, were stored in alcohol and later cleared and permanently mounted.

From approximately 10,000 mammals and 1,500 birds examined, 45 species of fleas were collected. Twenty-five species of mammals and 3 of 247 species and subspecies of birds examined were infected with fleas. Although many carnivores were examined and found infested with fleas, the majority of mammals investigated were rodents and rabbits.

Examinations of numerous bird nests and mammal burrows (nests) yielded fleas occasionally, but the data are included in this list only if the resident species was definitely known. It should be noted here that the composition of the flea fauna

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within a nest probably undergoes alteration upon the occupation of the nest by visiting host species. The above eventuality might possibly broaden the range of the flea fauna of the resident host as well as that of the nest. As indicated by the host-flea list, there exists a more or less free interchange of fleas between many of the host species, and quite often it becomes difficult to determine whether or not a preferred host exists for a given species. These data also indicate that there are some flea species that have a very restricted host range, being almost host specific. However, the authors feel that as more information is accumulated, the known host range of many of the seemingly relatively host specific species will be expanded.

Seasonal fluctuations in the composition of flea populations on mammalian hosts have been shown by Holdenried et al (1951) in California, and by Parker (1958) in the Great Salt Lake Desert. Also, seasonal fluctuations in the composition of the flea fauna in nests have been shown by Howell (1957). These data indicate that the periodicities of the environment influence the rate of interchange of a given species from host to host. The host range of a given species would be greater during periods when the flea populations are high. In order to accomplish a more nearly complete survey and to study the relationship between fleas and their hosts, it was necessary to study seasonal fluctuations by collecting fleas successively during every season.

A single flea taken from a Rocky Mountain downy woodpecker, *Dendrocopos pubescens leucurus* (Hartlaub) was recently described by Holland and Loshbaugh (1958) and named *Ornithophaga nearctica,* and assigned to Leptopsyllidae, subfamily Amphipsyllinae. This specimen is the only male known in its genus which, incidentally, was established by Mikulin (1956) for a single female taken from a "trydactylous woodpecker," *Picoides tridactylus* (Linn.) in U.S.S.R.

Identity of the North American species of *Pulex* having been questioned, extreme care was exercised in the identification of the available material. Male genitalia compared very favorably with the figure and description of *P. irritans* L. by Smit (1958).

The following list presents the host-flea relationships observed and indicates the relative frequency of their occurrence. Numerical symbols representing these frequencies appear in parentheses after each species in which only infested hosts were considered. These values were arbitrarily designated as follows: (4) common, designating that a given flea species was recovered from the host species on more than 50 percent of those examined; (3) frequent, the relationship occurred on between 30 and 50 percent; (2) occasional, it occurred on from 10 to 30 percent; and (1) rare, the species was recovered from less than 10 percent of the infested hosts examined.

From 86 specimens of little pocket mice, *Perognathus longimembris* (Coues), and 70 specimens of kangaroo mice, *Microdipodops megacephalus* Merriam, no fleas were removed. The total absence of fleas from these small rodents cannot be fully explained at this time, although Hubbard (1947) reported that few collected specimens of several species of *Perognathus* and of another subspecies of *Microdipodops megacephalus* were infested by fleas, and attributed the low recorded densities to the dusting habit which these animals commonly exhibit. This habit may be largely responsible for the low flea indices of these species in this area as well. However, other factors are considered. In this area, this habit does not completely free kangaroo rats, or some of the other sand dune-dwelling mice, from flea infestation,

but may tend to reduce the numbers of fleas per animal.

A characteristic ecological restriction of the mice to small, discontinuous sets of vegetated sand dunes as suggested by Mr. E. Dean Vest,* (unpublished manuscript) might tend to restrict the possibility of contact of the little pocket mice and the kangaroo mice with other rodents, thereby reducing accidental transfers of fleas. According to his studies, these animals occupy specific portions of the sand dune complex and consequently seldom contact other species. Vest also suggested that the relatively small size of the two animals, and of their burrow openings, physically restricts larger nocturnal animals from entering, particularly the wide-ranging deer mice, *Peromyscus maniculatus* (Wagner) and the dominant Ord's kangaroo rats, *Dipodomys ordii* Woodhouse. He has frequently observed deer mice entering accessible burrows of most other rodent species, and this would partially explain the mixed flea fauna (see list of fleas associated with *Peromyscus maniculatus*) of this animal.

Although the above-mentioned factors may partially explain a limited degree of transfer of fleas from other rodents to little pocket mice and kangaroo mice, the question of why they do not harbor a species of their own remains, for the moment, unanswered.

Records from data gathered by Mr. George Loshbaugh† are included in the list. Host-flea relationships recorded by him, which have not since been confirmed by the authors, are preceded by an asterisk.

Mammalian hosts are listed in their taxonomic order as given by Miller and Kellogg (1955); and to facilitate reference to the fleas, species are listed alphabetically.

HOST-FLEA LIST

MAMMALS

Family Leporidae (Hares and Rabbits)

Lepus californicus Gray . . . Black-tailed jackrabbit
1. *Cediopsylla i. inaequalis* (Baker) (4)
*2. *Meringis parkeri* Jordan (1)
3. *Odontopsyllus dentatus* (Baker) (2)
4. *Pulex irritans* Linnaeus (1)
5. *Thrassis aridis campestris* (Prince) (1)
6. *T. bacchi gladiolis* (Jordan) (1)

Sylvilagus nuttallii (Bachman) . . . Nuttall cottontail
1. *Cediopsylla i. inaequalis* (Baker) (4)
2. *Odontopsyllus dentatus* (Baker) (2)
3. *Thrassis bacchi gladiolis* (Jordan) (1)

Sylvilagus audubonii (Baird) . . . Audubon cottontail
1. *Cediopsylla i. inaequalis* (Baker) (4)
2. *Odontopsyllus dentatus* (Baker) (2)

Family Sciuridae (Squirrels)

Citellus townsendii (Bachman) . . . Townsend ground squirrel
*1. *Opisocrostis tuberculatus* (Baker) (1)
*2. *Thrassis francisi* (C. Fox) (1)
3. *Hoplopsyllus anomalus* (Baker) (1)

Citellus variegatus (Erxleben) . . . Rock squirrel
1. *Hoplopsyllus anomalus* (Baker) (3)
2. *Opisodasys keeni* (Baker) (1)

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Citellus leucurus (Merriam) . . . Antelope ground squirrel
1. *Foxella ignota* (Baker) (1)
2. *Hoplopsyllus anomalus* (Baker) (4)
3. *Malaraeus sinomus* (Jordan) (1)
4. *Megarthroglossus smiti* Mendez (1)
5. *Meringis dipodomys* Kohls (1)
6. *M. parkeri* Jordan (1)
7. *Orchopeas leucopus* (Baker) (1)
8. *Oropsylla idahoensis* (Baker) (1)
9. *Pulex irritans* Linnaeus (1)
10. *Rhadinopsylla fraterna* (Baker) (1)
11. *R. heiseri* (McCoy) (2)
12. *Thrassis bacchi gladiolis* (Jordan) (4)

Eutamias minimus (Bachman) . . . Least chipmunk
1. *Anomiopsyllus amphibolus* Wagner (1)
2. *Catallagia neweyi* Holland and Loshbaugh (1)
3. *Meringis dipodomys* Kohls (1)
4. *Monopsyllus eumolpi cyrturus* (Jordan) (4)
5. *M. w. wagneri* (Baker) (1)
6. *Thrassis bacchi gladiolis* (Jordan) (1)

Eutamias dorsalis (Baird) . . . Cliff chipmunk
1. *Monopsyllus eumolpi cyrturus* (Jordan) (4)

Family Geomyidae (Pocket Gophers)

Thomomys bottae (Eydoux and Gervais) . . . Botta pocket gopher
1. *Foxella ignota* ssp. (Baker) (4)
2. *F. u. utahensis* (Wagner)* (4)
3. *Hoplopsyllus anomalus* (Baker) (1)

Family Heteromyidae (Pocket Mice, Kangaroo Mice and Kangaroo Rats)

Perognathus parvus (Peale) . . . Great Basin pocket mouse
1. *Meringis parkeri* Jordan (1)

Perognathus formosus Merriam . . . Long-tailed pocket mouse
1. *Carteretta carteri clavata* Good (2)
2. *Malaraeus sinomus* (Jordan) (2)
3. *Meringis parkeri* Jordan (1)

Dipodomys ordii Woodhouse . . . Ord kangaroo rat
1. *Foxella* sp. (1)
2. *Hoplopsyllus anomalus* (Baker) (1)
3. *Malaraeus sinomus* (Jordan) (1)
4. *M. telchinum* (Rothschild) (1)
5. *Meringis dipodomys* Kohls (2)
*6. *M. jewetti* Hubbard (1)
7. *M. parkeri* Jordan (4)
*8. *Monopsyllus e. exilis* (Jordan) (1)
9. *M. w. wagneri* (Baker) (1)
10. *Orchopeas leucopus* (Baker) (1)
11. *O. sexdentatus* (Baker) (1)
12. *Thrassis aridis campestris* (Prince) (3)
13. *T. bacchi gladiolis* (Jordan) (1)

Dipodomys microps (Merriam) . . . Chisel-toothed kangaroo rat
1. *Catallagis neweyi* Holland and Loshbaugh (1)
2. *Meringis dipodomys* Kohls (2)
3. *M. parkeri* Jordan (4)
4. *Monopsyllus w. wagneri* (Baker) (1)
5. *Odontopsyllus dentatus* (Baker) (1)
6. *Orchopeas leucopus* (Baker) (1)
7. *O. sexdentatus* (Baker) (1)
8. *Thrassis aridis campestris* (Prince) (2)

* The authors have followed Hubbard (1947) in their determinations of this species, but wish to point out the need for a more critical consideration of the genus based on morphology as well as the fact that in this study both *F. ignota* and *F. u. utahensis* have commonly occurred on the same host.

Family Cricetidae (Mice and Rats)

Reithrodontomys megalotis (Baird) . . . Western harvest mouse
- *1. *Catallagia neweyi* Holland and Loshbaugh (1)
- 2. *Epitedia stanfordi* Traub (1)
- 3. *Malaraeus sinomus* (Jordan) (4)
- 4. *M. telchinum* (Rothschild) (2)
- 5. *Meringis parkeri* Jordan (1)
- 6. *Monopsyllus w. wagneri* (Baker) (3)
- 7. *Orchopeas leucopus* (Baker) (4)
- 8. *O. sexdentatus* (Baker) (1)
- 9. *Stenistomera macrodactyla* Good (1)

Peromyscus crinitus (Merriam) . . . Canyon mouse
- 1. *Anomiopsyllus amphibolus* Wagner (3)
- 2. *Atyphloceras* sp. (1)
- *3. *Callistopsyllus* sp. (1)
- *4. *Epitedia stanfordi* Traub (1)
- 5. *Malaraeus sinomus* (Jordan) (4)
- 6. *M. telchinum* (Rothschild) (2)
- 7. *Megarthroglossus smiti* Mendez (2)
- *8. *Meringis dipodomys* Kohls (1)
- 9. *Monopsyllus eumolpi cyrturus* (Jordan) (1)
- 10. *M. w. wagneri* (Baker) (3)
- 11. *Orchopeas leucopus* (Baker) (2)
- 12. *O. sexdentatus* (Baker) (2)
- 13. *Peromyscopsylla hesperomys adelpha* (Rothschild) (1)
- 14. *Rhadinopsylla goodi* (Hubbard) (1)
- 15. *Stenistomera alpina* (Baker) (2)
- 16. *S. macrodactyla* Good (2)
- 17. *Thrassis bacchi gladiolis* (Jordan) (1)

Peromyscus maniculatus (Wagner) . . . Deer mouse
- 1. *Anomiopsyllus amphibolus* Wagner (2)
- *2. *Callistopsyllus* sp. (1)
- 3. *Catallagia neweyi* Holland and Loshbaugh (1)
- *4. *Epitedia stanfordi* Traub (2)
- 5. *Hoplopsyllus anomalus* (Baker) (1)
- *6. *Hystrichopsylla linsdalei* Holland (1)
- 7. *Malaraeus sinomus* (Jordan) (4)
- 8. *M. telchinum* (Rothschild) (1)
- *9. *Megabothris abantis* (Rothschild) (1)
- *10. *Megarthroglossus becki* Tipton & Allred (1)
- 11. *M. smiti* Mendez (1)
- *12. *Meringis dipodomys* Kohls (1)
- *13. *M. parkeri* Jordan (2)
- 14. *Monopsyllus w. wagneri* (Baker) (4)
- 15. *M. eumolpi cyrturus* (Rothschild) (1)
- 16. *M. exilis exilis* (Jordan) (1)
- 17. *Orchopeas leucopus* (Baker) (1)
- 18. *O. sexdentatus* (Baker) (1)
- 19. *Opisodasys keeni* (Baker) (2)
- 20. *Peromyscopsylla hesperomys adelpha* (Rothschild) (1)
- 21. *Rhadinopsylla heiseri* (McCoy) (1)
- 22. *R. sectilis* (J. and R.) (1)
- 23. *Stenistomera macrodactyla* Good (1)
- 24. *Thrassis aridis campestris* (Prince) (1)
- 25. *T. bacchi gladiolis* (Jordan) (2)

Peromyscus truei (Shufeldt) . . . Pinyon mouse
- *1. *Anomiopsyllus amphibolus* Wagner (2)
- *2. *Atyphloceras* sp. (1)
- *3. *Carteretta carteri clavata* Good (1)
- 4. *Epitedia stanfordi* Traub (1)
- 5. *Malaraeus sinomus* (Jordan) (3)
- 6. *M. telchinum* (Rothschild) (1)
- 7. *Megarthroglossus smiti* Mendez (1)

8. *Meringis parkeri* Jordan (1)
9. *Monopsyllus w. wagneri* (Baker) (2)
10. *Orchopeas leucopus* (Baker) (3)
11. *O. sexdentatus* (Baker) (1)
12. *Stenistomera alpina* (Baker) (1)
13. *Thrassis aridis campestris* (Prince) (1)
14. *T. bacchi gladiolis* (Jordan) (2)

Onychomys leucogaster (Wied-Neuwied) . . . Grasshopper mouse
1. *Dactylopsylla comis* Jordan (1)
2. *Foxella ignota* (Baker) (2)
3. *Hoplopsyllus anomalus* (Baker) (1)
4. *Malaraeus telchinum* (Rothschild) (1)
5. *Meringis parkeri* Jordan (4)
6. *Monopsyllus e. exilis* (Jordan) (4)
7. *M. w. wagneri* (Baker) (1)
8. *Peromyscopsylla hesperomys adelpha* (Rothschild) (1)
9. *Rhadinopsylla heiseri* (McCoy) (1)
10. *Thrassis aridis campestris* (Prince) (2)
11. *T. bacchi gladiolis* (Jordan) (3)

Neotoma lepida Thomas . . . Desert wood rat
1. *Anomiopsyllus amphibolus* Wagner (4)
*2. *Anomiopsyllus* sp. (1)
3. *Epitedia stanfordi* Traub (1)
4. *Malaraeus sinomus* (Jordan) (1)
5. *Megarthroglossus smiti* Mendez (4)
6. *Meringis dipodomys* Kohls (1)
7. *M. parkeri* Jordan (1)
8. *Monopsyllus eumolpi* (Rothschild) (1)
9. *M. w. wagneri* (Baker) (2)
10. *Opisodasys* sp. (1)
11. *Orchopeas leucopus* (Baker) (1)
12. *O. sexdentatus* (Baker) (4)
13. *Rhadinopsylla heiseri* (McCoy) (1)
14. *Stenistomera alpina* (Baker) (3)
15. *S. macrodactyla* Good (1)
16. *Thrassis aridis campestris* (Prince) (2)
17. *T. bacchi gladiolis* (Jordan) (2)

Neotoma cinerea (Ord) . . . Bushy-tailed wood rat
1. *Anomiopsyllus amphibolus* Wagner (2)
*2. *Anomiopsyllus* sp. (1)
3. *Diamanus montanus* (Baker) (1)
4. *Hystrichopsylla linsdalei* Holland (2)
5. *Megarthroglossus smiti* Mendez (2)
6. *Opisodasys* sp. (1)
7. *Orchopeas sexdentatus agilis* (Rothschild) (4)
8. *Stenistomera alpina* (Baker) (2)

Family Canidae (Foxes, Coyotes and Wolves)

Canis latrans Say . . . Coyote
1. *Pulex irritans* Linnaeus (4)

Vulpes macrotis Merriam . . . Kit fox
1. *Meringis parkeri* Jordan (1)
2. *Monopsyllus wagneri* (Baker) (1)
3. *Pulex irritans* Linnaeus (4)
4. *Thrassis bacchi gladiolis* (Jordan) (1)

Family Mustelidae (Weasels and Allies)

Taxidea taxus (Schreber) . . . Badger
1. *Pulex irritans* Linnaeus (2)

Spilogale gracilis Merriam . . . Spotted skunk
1. *Hoplopsyllus anomalus* (Baker) (1)
*2. *Megarthroglossus* sp. (1)
*3. *Orchopeas sexdentatus* (Baker) (1)

Family Felidae (Cats)

Lynx rufus Schreber . . . Bobcat

1. *Cediopsylla i. inaequalis* (Baker) (2)
2. *Hoplopsyllus anomalus* (Baker) (1)
3. *Stenistomera alpina* (Baker) (1)

BIRDS

Family Strigidae (Owls)

Bubo virginianus occidentalis Stone . . . Montana horned owl

1. *Foxella ignota recula* (J. and R.) (1)
2. *F. ignota* ssp. (1)
3. *F. u. utahensis* (1)

Speotyto cunicularia hypugaea (Bonaparte) . . . Western burrowing owl

1. *Pulex irritans* Linnaeus (2)

Family Picidae (Woodpeckers)

Dendrocopos pubescens leucurus (Hartlaub) . . . Downy woodpecker

1. *Ornithophage nearctica* Holland and Loshbaugh (1)

SUMMARY

Mammal and bird fleas of the Great Salt Lake Desert region are listed according to host. Fleas were collected from 25 of 49 species of mammals and 3 of 247 species and sub-species of birds examined. Of the rodents from the area only two, *Perognathus longimembris* (Coues), and *Microdipodops megacephalus* Merriam, were uninfested by fleas. An accounting of these rodents indicates that certain specific ecological factors may influence their ectoparasite fauna.

Fleas were collected during all months of the year over a period of 8 years. The data include those collected from nests of mammals and birds only if the nest occupant was definitely known.

A symbol designating relative frequency of occurrence of each host-flea relationship is also included in the list.

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The taxonomy of fleas of the genus *Thrassis* herein recorded is taken from the keys furnished by Harold E. Stark of the San Francisco Field Station of the U. S. Public Health Service, who was good enough to loan data from his forthcoming manuscript entitled *Fleas of Utah*. Fleas of the genera *Actenophthalmus, Micropsylla* and *Rectofrontia* are congeneric and placed in the genus *Rhadinopsylla,* based on a revision published by Morlan and Prince (1954).

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THE ECTOPARASITES OF SOME MAMMALS FROM THE CHIRICAHUA MOUNTAINS, ARIZONA*

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Between July 27 and August 17, 1957, we trapped 834 small mammals from the Chiricahua Mountains in southeastern Arizona to obtain quantitative data on the populations of lice and fleas found on the mammals living in a semi-arid area. The Chiricahuas are an isolated group of mountains which rise abruptly from the desert floor at an elevation of 4,000 feet to a height of 9,795 feet. These mountains cover an area of about 40 miles long and 20 miles across at their widest part.

The areas where trapping was done included grassland, oak woodland, pinyon-juniper, and Douglas fir-yellow pine associations. The desert grassland area is located between the towns of Rodeo and Portal on the east side of the mountains. The oak woodland is located along the lower reaches of Cave Creek between Portal and the American Museum of Natural History's Southwestern Research Station. The pinyon-juniper types are along the road between the research station and Onion Gap and the town of Paradise. The Douglas fir-yellow pine habitat is between Onion Gap and Rustlers Park near the top of the mountain.

The mammals taken from the desert grassland were Merriam's kangaroo rat (*Dipodomys merriami* Mearns), silky pocket mouse (*Perognathus flavus* Baird), desert pocket mouse (*Perognathus penicillatus* Woodhouse), Harris' antelope ground squirrel (*Citellus harrisii* (Audubon and Bachman)), spotted ground squirrel (*Citellus spilosoma* (Bennett)), white-throated wood rat (*Neotoma albigula* Hartley), southern grasshopper mouse (*Onychomys torridus* (Coues)), cactus mouse (*Peromyscus eremicus* Baird), and deer mouse (*P. maniculatus sonoriensis* (LeConte)).

From the oak woodland, which follows Cave Creek and its tributaries, we captured the brush mouse (*Peromyscus boylii* (Baird)), silky pocket mouse, a coati (*Nasua narica* (L.)) and the cliff chipmunk (*Eutamias dorsalis* (Baird)).

From the pinyon-juniper habitat we took the brush mouse, the rock mouse (*Peromyscus nasutus* (J. A. Allen)), the Mexican wood rat (*Neotoma mexicana* Baird) and the cliff chipmunk. A white-tailed deer (*Odocoileus virginianus* Zimmerman) that had been taken from this habitat was also examined. The Mexican wood rat and cliff chipmunk were taken at the upper edge of the pinyon-juniper habitat where there is a close association with the Douglas fir-yellow pine association. From this latter habitat we captured the deer mouse (*Peromyscus maniculatus rufinus* (Merriam)), western harvest mouse (*Reithrodontomys megalotis* (Baird)), rock mouse, brush mouse, Mexican wood rat and the cliff chipmunk.

The animals were collected by setting out lines of snap traps through the several habitat types. The traps were placed one to the station, with stations about 30 feet apart. The traps were baited with peanut butter and left out for 3 days. A total

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of 25,955 trap days were involved. The density of the small mammal populations varied considerably from one association to another. It required 26 trap days on the desert grassland, 102 trap days in the oak woodland, 74 trap days in the pinyon-juniper and 15 trap days in the Douglas fir-yellow pine associations to capture one small mammal. This suggests that the small mammal population densities on the Douglas fir-yellow pine association were quite high, the populations on the desert grassland were moderate, while those in the oak woodland and the pinyon-juniper associations were quite low. The traps were examined early in the morning and each animal captured was placed in a separate pint-size polyethylene home freezer bag to prevent loss and transfer of parasites from one animal to another (Cook, 1954b). Each bag was numbered and each skin returned to this original bag after the host was examined, skinned and the skin dried. Most of the fleas were captured during the skinning process. The recovery of the lice and some of the fleas was accomplished by placing the whole skin in a solution of trypsin followed by KOH (Cook, 1954a). The ectoparasites were sexed, aged to instar, and counted after being recovered.

This study was made possible by the cooperation of the American Museum of Natural History's Southwestern Research Station.

SIPHONAPTERA

The numbers of fleas taken do not represent total populations, since the techniques used did not prevent the escape of individuals from the host before they were taken from the traps and some were known to have escaped while the hosts were being examined. The figures, however, do give some idea as to the relative abundance, preferred host, and habitat for each species.

Anomiopsyllus nudatus (Baker). Sixteen male and 31 female fleas of this species were collected. Forty-two were taken from 18 white-throated wood rats, 3 from 8 Mexican wood rats and 2 from 217 Merriam's kangaroo rats. This suggests that the flea is primarily associated with the wood rats and to a lesser extent with the habitat.

Catallagia sp. Four specimens, 2 males and 2 females, were taken from deer mice and 1 male from a brush mouse. The hosts were taken from the Douglas fir-yellow pine association.

Diamanus montanus (Baker). Single male specimens were taken from the deer mouse and the rock mouse. Both were taken at the lower edge of the Douglas fir-yellow pine association. Hubbard (1947) lists this as a flea which is primarily found on ground squirrels and only accidentally on deer mice. No ground squirrels were observed in the area from which these were collected.

Echidnophaga gallinacea (Westwood). One male and 12 female specimens of this cosmopolitan flea were taken. Of these, 8 were from Merriam's kangaroo rat, 3 from Harris' ground squirrel and 1 specimen each from the white-throated wood rat and deer mouse. In all cases they were from hosts taken in the desert grassland association.

Epitedia wenmanni (Rothschild). Three female specimens were taken from the deer mice collected in the Douglas fir-yellow pine association.

Hystrichopsylla dippiei Rothschild. One male and 1 female of this very large flea were taken from the deer mouse and brush mouse. In both cases the mice were from the Douglas fir-yellow pine association.

Malaraeus sinomus (Jordan). Only 2 female specimens were recovered from the 190 specimens of brush mouse examined. They were from the pinyon-juniper association.

Monopsyllus eumolpi cyrturus (Jordan). The 31 cliff chipmunks were the hosts from which all 9 male and 11 female specimens of this species were taken. The flea seemed to be present wherever this host was found, which included the oak woodland, pinyon-juniper and Douglas fir-yellow pine associations.

Monopsyllus wagneri (Baker). This flea was taken only from the Douglas fir-yellow pine association on the top of the mountains. A total of 27 specimens, 7 males and 20 females, were taken from the 210 deer mice (*P. m. rufinus*) examined. None were taken from the other species of *Peromyscus* and none were taken from the 4 specimens of the deer mouse (*P. m. sonoriensis*) which inhabits the desert grassland.

Orchopeas sp. A single unidentifiable female, which was not *O. sexdentatus,* was collected from 1 of 8 Mexican wood rats taken in the upper edge of the pinyon-juniper association.

Orchopeas sexdentatus agilis (Rothschild). Two male specimens were taken from 18 white-throated wood rats. These came from the desert grassland association.

Orchopeas sexdentatus neotomae Augustson. Fourteen specimens, 8 males and 6 females, were taken from Mexican wood rats. All were from the upper edge of the pinyon-juniper association. It is interesting to note that in this area it was taken only from Mexican wood rats and not from the white-throated wood rats which were found only a few miles away, even though it had been reported from the latter host in Arizona (Hubbard, 1947). Only *O. s. agilis* was found on the white-throated wood rat. It would appear that physical habitat rather than the species of wood rat present might control the distribution of the several subspecies of this flea.

Peromyscopsylla adelpha (Rothschild). Six males and 1 female were taken from deer mice and 1 female from a Mexican wood rat. These were from the lower edge of the Douglas fir-yellow pine association.

Pleochaetis sibynus (Jordan). This flea, although one of the most common on the animals examined, is rare in collections. It was first described from a single specimen taken from a skunk at Paradise, Arizona. Although we trapped the Paradise area, we did not take it at this relatively low elevation. It was found entirely in the Douglas fir-yellow pine and upper pinyon-juniper associations where it was taken from brush mice (3 male and 10 female fleas), deer mice (32 male and 34 female fleas), Mexican wood rats (2 female fleas) and the western harvest mouse (2 female fleas). This suggests that it is a rodent flea and that the type specimen was a straggler, as are many of the fleas recorded from carnivores.

Pulex sp. Two female specimens were recovered from a young coati taken in the oak woodland association.

Stenoponia ponera Traub and Johnson. One male and 4 female specimens were collected from deer mice and 1 from a brush mouse. The hosts were taken in the Douglas fir-yellow pine association.

MALLOPHAGA

Damalinia parallelus (Osborn). A number of specimens were taken from the region of the udder on a white-tailed deer.

Trichodectes pallidus Piaget. A large population of this louse was found on a young coati.

ANOPLURA

In recent work on the population structures of ANOPLURA in Minnesota (Cook and Beer, 1955 and 1958) and from the coastal forest of Oregon (Beer and Cook, 1958), it was found that there was considerable difference in species composition and population structure of lice on the same host species in different geographical areas. It appeared that these differences might be attributable to climatic conditions. The present collection was made from the semi-arid southwest to test this hypothesis.

Enderleinellus longiceps Kellogg and Ferris. A single specimen of this tree squirrel louse was taken from a Merriam's kangaroo rat. Since the kangaroo rats were taken in a habitat at considerable distance from tree squirrel habitat, tree squirrels were scarce where present, and no tree squirrels were handled, it is difficult to account for this specimen.

Enderleinellus suturalis (Osborn). Four of 7 Harris' ground squirrels were infested with this louse. However, in contrast to the populations of *Neohaematopinus citellinus* Ferris, the average population was relatively large. The male and female Harris' ground squirrels averaged 83 and 97 lice each, the largest population being 235 individuals and the smallest a single specimen. These extremes were both found on males.

Fahrenholzia pinnata Kellogg and Ferris. This louse, which is found on many species of heteromyid rodents, was taken on about 88 and 71 percent of the 121 male and 93 female Merriam's kangaroo rats, respectively. Populations varied from 1 to 301 lice on the male kangaroo rats, with an average of 20.4. On the female kangaroo rats a maximum of 80 and an average of 10.7 lice were found. One male silky pocket mouse of the 7 examined had 2 lice of this species. No lice were taken from the 71 desert pocket mice examined, even though they were taken on the same desert grassland area as the kangaroo rats.

Hoplopleura arboricola Kellogg and Ferris. All 31 of the cliff chipmunks examined were infested. The infestations varied from 25 to 551 with averages of 179 and 260 lice for the male and female hosts, respectively.

Hoplopleura ferrisi Cook and Beer. Sixty-six of the 193 brush mice were found to be infested with an average of 12.3 lice per infested host. Eleven of the 19 rock mice were found to be infested with average populations of 23 and 38 lice per infested male and female hosts respectively.

Two of the 13 cactus mice were infested with single specimens of *H. ferrisi*. Since this louse was also collected from cactus mice taken near Wilna, New Mexico we conclude that this is also a normal host for this parasite.

A single specimen was taken from 1 of the 18 white-throated wood rats and another from 1 of 214 Merriam's kangaroo rats examined. These were probably cases of straggling.

Hoplopleura onychomydis Cook and Beer. Nine of the 13 grasshopper mice examined were infested with this louse. The average population per infested host was 12 and 8 lice for the male and female hosts, respectively. The populations varied from 1 to 33 lice.

Neohaematopinus citellinus Ferris. Three of the 7 Harris' ground squirrels had from 1 to 5 lice with an average of 3.7 each.

Neohaematopinus neotomae Ferris. Eight of 18 white-throated wood rats and 3 of 8 Mexican wood rats examined harbored this louse. The populations varied from 1 to 8 specimens with an average of 5 per host.

Neohaematopinus pacificus Kellogg and Ferris. This louse was taken only from the cliff chipmunk. Twenty-two of 31 cliff chipmunks examined were infested with populations varying from 1 to 47 lice each. The populations averaged 15 and 7.8 lice per infested male and female host respectively.

Polyplax auricularis Kellogg and Ferris. This louse was found on 3 of the 4 species of *Peromyscus* taken in the Chiricahua Mountain area. The fourth species, the cactus mouse, did not harbor this louse. However, this *Polyplax* was taken from the cactus mouse in the vicinity of Wilna, New Mexico. It was also taken from 6 of 13 grasshopper mice examined. Only 3 specimens were taken from a deer mouse (*P. m. rufinus*), though 210 animals were examined. None was taken from the single western harvest mouse and 4 deer mice (*P. m. sonoriensis*).

Thirteen percent of the male and 14 percent of the female brush mice were infested. The infestations varied from 1 to 27, with an average of 5.7, lice on the males; and 1 to 11, with an average of 3.4, lice on the females. Five of 19 rock mice were infested with populations which varied from 1 to 7 with an average of 2.4 per infested host. Seven of 13 southern grasshopper mice were infested. The populations varied from 3 to 14 with an average of 7.9 lice per host.

DISCUSSION

For the most part the fleas were more closely related to the habitat than to a host species. In this collection 4 species were found only in the desert grassland, 1 only in the oak woodland, 2 only in the pinyon-juniper association, and 9 only from the Douglas fir-yellow pine. Only 2 species were found regularly in more than one habitat. *Monopsyllus eumolpi* was found on the cliff chipmunk regardless of habitat, and *A. nudatus* on wood rats in both the desert grassland and upper edge of the pinyon-juniper association. At least during late summer, fleas were more abundant and more species available on the cooler and more moist mountain tops than on the desert grasslands, oak woodland, and pinyon-juniper associations.

In contrast to the rather close relationship with the physical habitat, several fleas were found on more than 1 host species. Nine species were found on but a single host species, 5 on 2, 1 on 3, and 2 on 4.

No fleas were found on the 71 specimens of the desert pocket mouse, 7 specimens of the silky pocket mouse, or the single spotted ground squirrel. The 214 Merriam's kangaroo rats examined harbored altogether but 2 *A. nudatus* and 8 *E. gallinacea*. The first is a flea found primarily on wood rats, while the latter has a wide number of known hosts. None of the fleas considered to be regularly associated with kangaroo rats were found.

Eleven species of Anopleura were taken from 11 species of small mammals. Six species of lice were found regularly on a single host species, 3 on 2, 1 on 3 and 1 on 5. In only 2 cases was a species of louse found regularly on 2 genera of hosts.

The rate of infestation of the several species of lice on their respective hosts varied from 0 percent for *H. hesperomydis* on the 210 deer mice taken at high elevation to 100 percent for *H. arboricola* on the 31 cliff chipmunks. *Hoplopleura ferrisi* was taken from 33.0 percent of the male and 35.5 percent of the female brush mice (see table I). A Chi-square value of 0.00011 with three degrees of freedom

TABLE I. *Population characteristics of lice on some rodents from the Chiricahua Mountains in southeastern Arizona.*

Parasite	Host	No. of hosts examined	Percent infested	Total lice	Lice per infestation			Structure of louse populations in percent				
					Max.	Min.	Av.	Adults		Developing instars		
								Males	Females	1st	2nd	3rd
E. suturalis	*C. harrisii*, male	2	50.0	83	83	83	83.0	25.3	50.6	6.0	1.2	14.4
	C. harrisii, female	5	60.0	292	285	1	97.0	18.2	48.0	11.3	5.2	17.5
F. pinnata	*D. merriami*, male	121	87.6	2164	301	1	20.4	19.7	27.1	24.1	13.6	15.4
	D. merriami, female	93	71.0	707	80	1	10.7	20.4	24.9	29.6	12.8	13.0
H. arboricola	*E. dorsalis*, male	19	100	3401	540	29	179	10.9	10.5	35.1	24.9	18.5
	E. dorsalis, female	12	100	3115	551	25	260	12.3	12.0	40.3	21.6	14.7
H. ferrisi	*P. boylii*, male	100	33.0	422	76	1	12.8	16.7	21.8	40.7	18.0	5.4
	P. boylii, female	93	33.5	387	71	1	11.8	17.6	23.8	28.2	16.0	4.4
N. pacificus	*E. dorsalis*, male	19	73.7	212	47	1	15.1	17.5	18.4	31.6	19.8	12.7
	E. dorsalis, female	12	66.7	63	20	1	7.8	17.2	12.5	44.5	11.1	14.3
P. auricularis	*P. boylii*, male	100	13.0	74	27	1	5.7	23.0	41.9	23.0	10.8	1.4
	P. boylii, female	93	14.0	44	11	1	3.4	36.4	38.7	15.8	6.8	2.3

is strong evidence that there is no significant difference in the infestation rate in the 2 sexes of this host. This rate of infestation and the lack of apparent difference in the rate between the sexes agrees with data from *H. hesperomydis* on the deer mouse in southern Minnesota (Cook and Beer, 1958), although it differs in both respects from similar data from western Oregon (Beer and Cook, 1958) and northern Minnesota (Cook and Beer, 1955). Some 33.7 percent of the deer mice from southern Minnesota were infested. On the other hand, male and female deer mice from Oregon had infestation rates of 62 and 52 percent, respectively, and those from northern Minnesota had infestation rates which varied from 18.5 to 58 percent for male and from 11.4 to 25 percent for female hosts. The louse *P. auricularis* was taken from several hosts, but only the deer mouse and brush mouse samples were large enough to consider. Only 1 deer mouse out of 210 was infested, while 13 and 14 percent of the male and female brush mice were infested, respectively. A Chi-square value of 0.045 with 3 degrees of freedom indicates that there is no significant difference in rate of infestation of the 2 sexes of brush mice. These rates are somewhat at variance with our previous studies. No lice of this species were taken from the 2 subspecies of deer mice examined from Minnesota (Cook and Beer, 1955 and 1958) while more male than female deer mice were infested in the sample from western Oregon (Beer and Cook, 1958). In the latter sample 25 percent of the male and 13 percent of the female deer mice were infested.

The Merriam's kangaroo rat was the only other infested host taken in large enough numbers to warrant the examination of infestation rates. Here the louse *F. pinnata* was found on 87.6 and 71.0 percent of the male and female kangaroo rats, respectively. A Chi-square value of 8.201 with three degrees of freedom suggests that there is a real difference in infestation rate between the sexes.

The size of the louse populations varied considerably among the several host species examined (see table I). In most cases the number of infested hosts was too small to be more than an indication of what the average population was. In only one case was the size of the population significantly different between the sexes of the host. The average population of *F. pinnata* on the male and female Merriam's kangaroo rat was 20.4 and 10.7, respectively. A "t" value of 2.28 with 172 degrees of freedom is significant at the 0.05 level.

The average population size of 12.2 *H. ferrisi* per infested brush mouse is very similar to the figures for *H. hesperomydis* on deer mice in western Oregon (Beer and Cook, 1958) and from northern Minnesota (Cook and Beer, 1955), though higher than the 5.8 found on the deer mice in southern Minnesota (Cook and Beer, 1958).

In those cases where 200 or more lice of a species were taken from a host species an analysis of the population structure was made. The sex ratio of adult lice varied from species to species, but in no case did the ratios vary significantly between the populations on the two sexes of host. As reported in other studies (Craufurd-Benson, 1941; Vysotskaia, 1950) there were usually more female than male lice in the population. The greatest discrepancy was found with *E. suturalis* on the Harris' ground squirrel, where there were only 29.0 percent males. About 43 percent of the adult *F. pinnata* and *H. ferrisi* were males. The adult populations of *N. pacificus* and *H. arboricola* on the cliff chipmunk were composed of 48.3 and 50.8 percent males, respectively (see table I).

There was considerable difference in the ratio of adult female lice to the number of immatures present in the several populations (see table I). In 2 cases, *F. pinnata* on the kangaroo rat and *H. ferrisi* on the rock mouse, there were significantly different age ratios on the male and female hosts. On the former there were 1.96 immature forms per adult female on the male host and 2.45 immatures per adult female on the female hosts. A Chi-square value of 17.939 with 3 degrees of freedom is highly significant. A Chi-square value of 32.642 with 3 degrees of freedom indicates that the 0.37 and 4.23 immatures per adult female *H. ferrisi* on the male and female rock mice is also a significant difference. However, since this latter was from a relatively small sample and we found no difference in the much larger sample from the brush mouse, we feel that this needs further investigation. The lowest ratio of immatures to adult females was 0.65 for *E. suturalis* on the Harris' ground squirrel. A ratio of 2.71 was found for *H. ferrisi* on the brush mouse, 3.83 for *N. pacificus*, and 6.92 for *H. arboricola* on the cliff chipmunk. In all cases the immature population was composed primarily of first instars with relatively few third instars.

Two hosts had populations of 2 species of lice which could be used to determine whether the different species were found on the host for similar reasons. Cole's (1949) coefficient for interspecific association was calculated to determine similarities in requirement. *Hoplopleura ferrisi* and *P. auricularis* were both found on the brush mouse. A coefficient of 0.182 ± 0.133 suggests that there is but little though probably significant amount of similarity in requirements. A value of 0.525 ± 0.448 for these same lice on the rock mouse suggests possibly a stronger association. In both cases the 2 species of lice are distributed among the hosts primarily independently of each other. These data are somewhat at variance with those from these same louse species on the deer mouse in Oregon (Beer and Cook, 1958) where a coefficient of 0.007 ± 0.016 was obtained.

One of the unexpected findings of this study was the lack of lice of the genus *Hoplopleura* on the deer mice (*P. m. rufinus*) from the higher elevations. The sample of 210 hosts is large enough that it should be a reasonable representation of the population, and we might well assume that the deer mice from the high elevations have no *Hoplopleura*. The deer mice (*P. m. sonoriensis*) from Williams, Arizona, are infested with *H. hesperomydis*. It thus seems probable that since the populations of the deer mouse (*P. m. sonoriensis*) are reasonably continuous over the desert, that lice of the genus *Hoplopleura* are to be found wherever it is found. There is a large area of habitat, the oak woodland and the pinyon-juniper associations, which separate these 2 subspecies of deer mice (Hoffmeister, 1956; Hoffmeister and Goodpaster, 1954). The mice of the genus *Peromyscus* which occupy these areas are hosts to *H. ferrisi* and not *H. hesperomydis*.

There remain three possible explanations for the absence of the genus *Hoplopleura* from the deer mice from the upper areas of the Chiricahuas: (1) This species of mouse had not been exposed to infestations of *H. hesperomydis;* (2) none of the *Hoplopleura* from this region were adapted to life on it; and (3) this mouse once was infested, but later lost its infestation.

The first of these hypotheses does not seem probable. However, collections from this subspecies of deer mouse from the tops of the nearby mountain ranges might be very revealing.

The second hypothesis is possible and could be checked under laboratory conditions. This would mean that there is a higher degree of host specificity for these lice than is generally encountered.

It seems more probable that this population of *Peromyscus maniculatus* was once infested with *Hoplopleura hesperomydis* (Osborn) and subsequently lost them. This louse was taken from *Peromyscus maniculatus* near Williams, Arizona, a record which appears to rule out climatic isolation. Since the mouse population is limited to the top of the Chiricahua Mountains, it is conceivable that at times it has been reduced to so few animals that the louse population could not maintain itself.

SUMMARY

Altogether, 834 mammals were collected from the Chiricahua Mountains in southeastern Arizona and examined for fleas and lice. Sixteen species of Siphonaptera, 2 species of Mallophaga and 11 species of Anoplura were collected.

The fleas showed more relationship in their distribution to the physical habitat than to the host species. Most of the lice, on the other hand, were highly host specific.

The size of louse infestations varied from host species to host species, and on the Merriam's kangaroo rat between the sexes of the host.

The sex ratio varied from 29.0 percent males in *E. suturalis* to 50.8 percent in *H. arboricola*. In no case did the sex ratio of lice vary with the sex of the host.

The number of immature lice per adult female varied from 0.37 for *H. ferrisi* on the male rock mouse to 6.92 for *H. arboricola* on the cliff chipmunk. There were more immature lice per adult female louse on the female Merriam's kangaroo rats than on the males.

There was a significant positive interspecific association between *H. ferrisi* and *P. auricularis* on brush and rock mice.

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RESEARCH NOTES

OBSERVATIONS ON HOST PERCEPTION IN FLEAS

As part of a research project supported by the Research Foundation of State University of New York, some preliminary experiments were conducted on the ability of fleas to perceive the presence of a host and to distinguish between 2 different potential hosts.

The perception experiments were performed with the common and non-host-specific flea, *Ctenophthalmus p. pseudagyrtes* Baker. As a host we used an eastern chipmunk, *Tamias striatus*, a small mammal upon which this flea commonly occurs in nature. Tests were run by placing the flea on a grid card marked off into 1-inch squares, at varying distances from the host, which was placed in an open-mesh trap small enough to prevent it from moving about freely.

Experiments at distances of from 4 to 10 inches between flea and host produced entirely negative results. Of 33 fleas tested at these distances, 17 failed to move at all, and 16 leaped about at random, not reaching the host. Length of leaps in this species ranged from 2 to 7 inches.

At 3 inches between flea and host, 1 of 4 fleas reached the host after its first leap carried it diagonally to within 2 inches of the host. At 2 inches, 3 of 4 fleas leaped directly upon the host, while the 4th did not move at all.

These few data appear to indicate that this flea is unable to perceive the presence of a host the size of a chipmunk at distances exceeding 2 inches. Since this flea normally occurs on small mammals which spend much of their time in burrows, (e. g. *Blarina brevicauda, Pitymys pinetorum*, and the chipmunk), its limited perceptive ability is probably no handicap under ordinary circumstances.

Experiments to determine the ability of the flea to recognize a specific host were carried on with *Cediopsylla simplex* (Baker), a host-specific rabbit flea. The grid card setup, as described above, was used in these experiments, except that the preferred host, a cottontail rabbit, *Sylvilagus floridanus*, was placed at one end of the grid card and an opossum, *Didelphis marsupialis*, was placed an equal distance away at the opposite end. In one trial, the human arm was used as the alternate possibility. Nine of 22 released fleas failed to leap. Distances from flea to host at release were varied from 7 to 10 inches. None of the 13 fleas which leaped went directly to the host, although leaps varied from 2 to 10 inches, and 1 flea landed on the rabbit after a 7-inch leap. In all cases, these long leaps appeared to be quite random. Five of the 13 fleas reached the rabbit within the 3 minutes allotted to each trial, but none leaped upon the opossum or the human, despite the fact that several landed within 2 inches of these potential hosts. Although admittedly preliminary, these data indicate an apparent ability to distinguish between a preferred host and another mammal of equal size at short distances.—ALLEN H. BENTON, ROBERT CERWONKA AND JOSEPH HILL, *State University of New York, College for Teachers, Albany.*

THE PARAMPHISTOME *MEGALODISCUS MICROPHAGUS* INGLES, 1936, FROM THE GIANT SALAMANDER *DICAMPTODON ENSATUS* (ESCHSCHOLTZ, 1833) FROM OREGON.

Seven trematodes identified as *Megalodiscus microphagus* Ingles, 1936 (Trans. Am. Micr. Soc. 55: 73–92), were recovered from the rectum of a larval *Dicamptodon ensatus* (Eschscholtz, 1833) taken from a small pond near Mill Creek, a tributary of the Alsea River, Benton County, Oregon. This trematode described by Ingles from *Bufo boreas* from Shasta County, California, has not been reported since. The trematodes from *Dicamptodon* are considerably larger than those from *Bufo*, but agree in all other respects with the original description. Dimensions of the specimens from *Dicamptodon* follow: length 6.8 to 8.4 mm, width 1.8 to 2.5 mm, oral sucker 0.67 to 0.80 by 0.57 to 0.68 mm, posterior sucker 1.70 to 1.80 mm in diameter, prepharynx 0.35 to 0.65 mm long, esophageal bulb 0.22 to 0.32 by 0.21 to 0.27 mm, anterior testis 0.66 to 1.01 by 0.46 to 0.68 mm, posterior testis 0.49 to 0.86 by 0.45 to 0.69 mm, ovary 0.24 to 0.36 by 0.30 to 0.38 mm, ova 82 to 90 by 41 to 56 microns. (Aided by research grant E-867 from the U. S. Public Health Service.)—JAMES E. MCCAULEY AND IVAN PRATT, *Department of Zoology, Oregon State College, Corvallis, Oregon.*

QUANTITATIVE RECOVERY OF HELMINTH EGGS FROM RELATIVELY LARGE SAMPLES OF FECES AND SEWAGE

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The methods of recovering helminth eggs from feces and sewage described in the past are too numerous to review in this report. Most of the methods used are designed for fecal survey studies and several of the more rapid and efficient procedures are said to yield reliable qualitative results. Several quantitative methods have been developed, but these sample the egg burden of relatively small quantities of feces or sewage, and/or involve flotation in concentrated salt solutions or other steps that are damaging to some important groups of helminth eggs.

The technique described in this report is not a rapid procedure and could not be used in epidemiologic survey work; however, it provides an efficient means of examining large quantities of material, being designed for the quantitative recovery of helminth eggs, particularly those of *Schistosoma mansoni*, from 20-liter samples of domestic sewage. In addition to sewage work, the technique has been used to obtain viable schistosome eggs from ground mouse livers and intestines for physiological studies, to concentrate eggs from feces for teaching purposes, to measure the total number of eggs in 10 g or less of feces from persons undergoing therapy, and to examine samples of river water for helminth egg pollution.

Description of the Method

The technique developed requires an especially constructed apparatus similar to the one illustrated in figures 1 and 2A and 2B. Through the use of this equipment, a large part of the suspended material is eliminated from the sewage or fecal suspension thereby leaving a relatively clear solution containing the majority of the helminth eggs. The apparatus consists of 3 main parts: The dispensing chamber with 10 outlets, mounted on a ring stand; a rack containing 10 glass jets with rubber tubing connections; the horizontal settling tray, consisting of 10 channels, each 1.3 cm wide, 62 cm long, and 2.5 cm deep. The jets are made of pyrex glass tubing (6 mm outside diameter, 10 cm long) by drawing out one end of each tube to a fine point in the flame of a gas-oxygen burner, and then grinding this end down with carborundum powder until an aperture of the proper diameter is obtained. Each jet delivers approximately 30 ml of water per minute when the head above the aperture is approximately 40 cm. The horizontal settling tray is constructed of clear plastic 3 mm thick. Note that at the inlet end of each channel there is a level plastic shelf located 0.6 cm below the upper edge of the channel and extending 2.5 cm towards the outlet end. Each outlet is also 0.6 cm lower than the upper edge of the channel. Overflow from all 10 channels meets in a common basin and leaves the tray through a plastic outlet tube.

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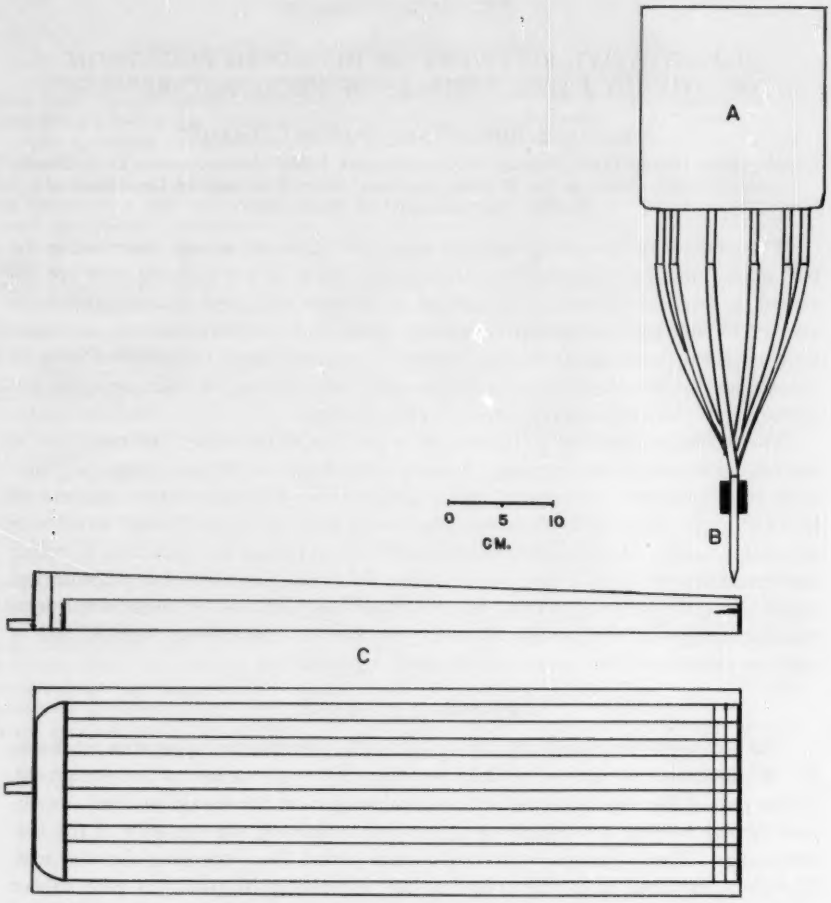

FIGURE 1. Apparatus designed for quantitative recovery of helminth eggs from fecal and sewage samples. A, dispensing chamber; B, glass jets; C, settling tray.

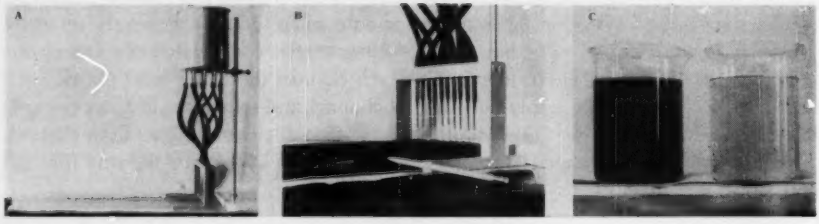

FIGURE 2. Photograph of apparatus showing A, complete unit; B, close-up of outlet jets; C, appearance of sewage samples before and after processing.

The procedure for the examination of sewage follows.

1. The sewage samples, either influent or effluent, are formalinized (1 percent) at the treatment plant and brought to the laboratory in 20-liter glass carboys. The suspended solids are allowed to settle in the carboy for 2 or more hours and then the supernatant fluid is withdrawn by means of a siphon until only 3 liters remain.

2. The sediment on the bottom of the carboy is re-suspended in the remaining 3 liters by swirling the carboy, and the resulting concentrate is strained through nylon net (90 threads to the inch) and then through bolting cloth (125 threads to the inch).

3. Portions of the strained sewage concentrate are poured into an electric blender and exposed to low speed mixing for 30 seconds. The electric blender breaks up the aggregates of bacteria and debris and retards flocculation. Blending at low speed does not damage a significant number of eggs.

4. The blended portions are poured, in turn, into the dispensing chamber of the separation apparatus. The separation apparatus is prepared to receive the sewage concentrate by filling the 10 channels of the horizontal tray with 1.0 percent NaCl solution. The sewage concentrate soon fills the glass jets and the jets deliver the fluid to each of the 10 saline-filled channels. The sewage concentrate, having a lower specific gravity than the saline, flows slowly along the surface of the saline. While doing so, the heavier particles in the concentrate, including most helminth eggs, gradually settle through this layer and become trapped in the lower, stationary layer of saline. Care must be taken to add the portions of sewage concentrate to the dispensing chamber one at a time so that the level in the dispensing chamber does not change enough to cause a marked fluctuation in rate of flow of the fluid from the jets. The 10 jets combined deliver a total of 300 ml of concentrate per minute, so that approximately 10 minutes is required for all of the fluid to pass through the apparatus. After all 3 liters of the concentrate has passed through the jets, the dispensing chamber is washed with several hundred ml of tap water to ensure that all traces of the concentrate including foam have left the dispensing chamber and passed through the jets. This clear water also serves to remove the sewage concentrate layer from the settling channels.

5. The channels are emptied into a beaker by tilting the settling tray, and each channel is washed with a stream of water from a wash bottle.

6. The channels are filled with a 2 percent NaCl solution and steps 3 to 5 are repeated. Figure 2C illustrates the extent to which suspended materials are removed following two passages through the apparatus.

(The helminth eggs contained in the saline are prepared for study and counting in the following manner.)

7. A filter pump and filter flask arrangement is used to provide vacuum for operation of a Büchner funnel (perforated plate approximately 111 mm in diameter). Aliquots of the egg-bearing saline from the settling tray are passed through filter paper (S and S, 404, 11 cm diameter). Each filter paper is prepared before use with parallel pencil lines spaced at 2- to 3-mm intervals. The eggs and other debris trapped on the paper should produce only a slight brownish discoloration of the paper. Too thick a layer of debris makes the papers difficult and tedious to examine. With practice the operator learns to judge how much fluid of a given turbidity must pass through a single paper to give the desired result, and, consequently, how many papers will be needed to filter all of the saline. Sewage influent samples, after processing, may require from 3 to 5 papers. Effluent samples usually

need only 1 or 2 papers.

8. The damp paper, after filtering, is placed in a shallow pan over 1 to 2 ml of 2.0 percent ninhydrin reagent. The pan is heated from beneath with a Bunsen burner until most of the ninhydrin has steamed away and the damp paper can be moved about in the pan with an applicator stick. The paper must not become dry. The ninhydrin stains some helminth eggs (e.g. *Schistosoma mansoni*) deep blue, but leaves the paper unstained. Staining can be omitted for such eggs as *Ascaris* and *Trichocephalus* which have naturally dark shells that contrast well with the background.

9. The damp paper is placed on a glass plate. Several ml of melted 2.0 percent agar-agar are poured over the paper and the agar is covered with a second glass plate. Gentle pressure is applied to the cover plate to remove trapped air. The agar solidifies rapidly and the plate can be examined immediately using a dissecting microscope (magnification 27×) and reflected light. If refrigerated, the plates will last for several days, but the ninhydrin stain usually begins to fade after 48 hours.

Quantitative Aspects of the Settling Chamber-Agar Plate Technique

Several experiments were done to establish the efficiency and consistency with which the technique recovers eggs from samples of water and sewage. In each of 5 trials, 100 freshly collected and perfectly normal *S. mansoni* eggs from mouse intestinal tissue were transferred to 3 liters of 1.0 percent formalin in tap water. These samples were strained through nylon mesh and bolting cloth, blended in the electric blender, passed through the various steps involving the settling tray, and the resulting sediments from the tray were prepared as agar plates in the usual manner (steps 2–9, above). The total numbers of eggs recovered on the paper in these 5 trials were 47, 53, 60, 56, and 67, or an average of 57.

The efficiency of the technique was tested in another manner. A 120-liter supply of sewage effluent (formalinized, 1 percent) was collected in a clean oil drum and the egg content of this supply was reinforced by adding human feces known to contain large numbers of helminth eggs, especially schistosome eggs. While stirring this preparation constantly, 1-liter and 18.9-liter samples were siphoned off alternately until there were on hand five 1-liter samples (in graduated cylinders) and four 18.9-liter samples (in glass carboys). The 1-liter samples were poured directly onto filter papers using the Büchner funnel procedure and the resulting papers were stained and mounted in agar. The 18.9-liter samples were allowed to stand overnight and then handled exactly as in steps 1–9 above. Total counts were made of *Ascaris, Trichocephalus,* and *Schistosoma* eggs on all of the papers. Two experiments of this type were done using 2 different 120-liter preparations of sewage.

Examination of the papers prepared from the 1-liter samples in both experiments gave the following results:

	Experiment 1			Experiment 2		
Sample Number	*Ascaris*	*Trichocephalus*	*Schistosoma*	*Ascaris*	*Trichocephalus*	*Schistosoma*
1	114	42	100	97	76	72
2	108	50	97	61	70	70
3	82	61	95	73	65	99
4	70	47	87	54	65	74
5	109	79	106	65	79	89
Average	97	56	97	62	71	81

Examination of the papers prepared from the 18.9-liter samples in both experiments gave the following results:

Sample Number	Experiment 1			Experiment 2		
	Ascaris	*Trichocephalus*	*Schistosoma*	*Ascaris*	*Trichocephalus*	*Schistosoma*
1	1046	506	1197	938	558	930
2	1238	622	1178	822	447	866
3	1348	469	1074	883	452	842
4	1249	460	1248	814	464	823
Average	1220	514	1174	864	480	865
Expected Recovery	1833	1058	1833	1172	1342	1531
Percentage Recovery	67	49	64	74	36	56

The expected recovery for each type of egg from the 18.9-liter samples was 18.9 times the number of eggs recovered from the 1-liter samples. The percentage recovery was the average recovery divided by the expected recovery times 100. These calculations are included in the above table. It should be noted that these experiments were done using an artificial sample containing many more eggs per liter than is usual under present conditions in Puerto Rico (see table I).

TABLE I. *Helminth eggs recovered from the San Juan sewage treatment plant, January 27, 1958.*

Source of the sample	Suspended solids (p.p.m.)	Helminth Eggs per 17 liters (not corrected to reflect efficiency of the technique)		
		Ascaris	*Trichocephalus*	*Schistosoma*
Influent	107	652	689	30
Effluent	47	48	235	4
Percentage Removal	66	93	66	87

By actual test, it was found that no eggs of the 3 types tested pass through the filter paper. By comparing the percentage recovery of schistosome eggs in the 2 calibration methods, it seems probable that few eggs are lost during the filtration, staining and plating steps of the technique. Consequently, the over-all efficiency of the technique, when used to recover helminth eggs from sewage, is approximately 70, 40, and 60 percent for *Ascaris, Trichocephalus,* and *Schistosoma,* respectively.

Recovery of Helminth Eggs in Sewage

Formalinized influent and effluent samples of sewage were obtained from the recently constructed primary sedimentation-type sewage treatment plant serving San Juan, Puerto Rico. These were composite samples of 17 liters, both whole samples consisting of nine 1.9-liter parts taken at hourly intervals from 8 a.m. to 4 p.m. The samples were processed according to the directions given previously and total counts were made of the eggs present on the filter papers. The results of these counts are presented in table I. Because of the relatively large size of the sample, it is possible to detect the presence of eggs occurring in extremely low density.

Quantitative Counts of S. mansoni *Eggs in Feces*

A specimen of feces was received from an individual just prior to the day that he began Fuadin treatment for schistosomiasis mansoni. A 10.1 g sample of this feces

was comminuted in 3 liters of 1.0 percent formalin, strained, and then processed in the usual manner (steps 3 to 9). A total of 1329 eggs was counted, or 131 eggs per wet gram. A sample of these feces of similar size was weighed, dried in a desiccator, and reweighed. The water content of this second sample, expressed as percent of dry weight, was found to be 71.2. It is estimated from these data that the feces contained approximately 455 eggs per dry gram. Seven days later, after having received 9.5 ml of Fuadin, the patient submitted another specimen. This specimen, which had a water content of 68.3 percent, yielded 27.5 eggs per wet gram or 86.7 eggs per dry gram. After 8 more days the patient had received a total of 19.5 ml of Fuadin. The feces, with a water content of 73.3 percent, contained 31.9 eggs per wet gram or 119.5 eggs per dry gram. The complete treatment required 41 days with the patient having received 59.5 ml of Fuadin. Thirty days after termination of treatment a final sample was examined. This sample, having a water content of 80.7 percent, contained no eggs.

A procedure of this type could be used to obtain an accurate measure of the total output of worm eggs from an individual for research purposes, provided all of his feces could be received over a period of time for weighing and sampling. The procedure might also be useful in determining the total worm egg output of groups of laboratory animals when egg output is an important indicator of drug activity, or a factor of interest in the physiology of a helminth.

Separation of Viable S. mansoni *Eggs from Tissues of Infected Mice*

Frequently it is useful to have large numbers of viable *S. mansoni* eggs free of tissue or other debris to use for physiological studies or other purposes. Coker and Lichtenberg (1956) described a procedure for separating schistosome eggs from the livers of infected hamsters. Their technique makes use of the differential rate of sedimentation of the eggs and tissue through sucrose solution. Ritchie and Berrios-Duran (personal communication, 1957) have developed a similar method employing saline. The idea, initiated by these men, of using dilute solutions of sugar or salt to facilitate separation of trematode eggs from other materials is included as described above as an indispensable part of the horizontal settling tray technique described in this paper.

Mouse livers (not more than 50 g wet weight) are comminuted in 3 liters of 1.0 percent saline. (Intestines can be used if they are opened longitudinally and washed.) The resulting suspension is strained through nylon net material and bolting cloth, blended in the electric blender, and poured into the dispensing chamber. The channels of the settling tray are filled with 1.5 percent saline solution. The liver tissue suspension flows over the surface of the 1.5 percent saline in the channels, and most of the contained eggs drop through the moving surface layer and become trapped in the saline. The saline-egg mixture is collected and blended, then passed over 2.0 percent saline in the settling tray. The second passage removes essentially all of the tissue, leaving clean, viable eggs. Thse eggs are collected by sedimentation in a graduated cylinder or other suitable container and are used immediately or stored in a refrigerator in prechilled 0.85 percent saline for subsequent use.

SUMMARY

The technique described provides an efficient quantitative means for recovering helminth eggs from 20-liter samples of sewage, 10-g samples of animal or human feces, or 50-g samples of comminuted animal tissue. It is not a rapid technique but it has been found to be an adaptable research tool for certain studies in the parasitological laboratory.

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SUGGESTIONS TO PROSPECTIVE AUTHORS

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It is essential that all regular papers appearing in The Journal of Parasitology be properly documented. To that end, all references to the literature properly cited in the text appear at the end in a list headed either Literature Cited or References, preferably the former. The heading Bibliography is not satisfactory, because it implies a complete list of works by an author or of the literature bearing on a particular subject. References to works not actually cited are not included.

Names of authors accompanying zoological names discussed or mentioned in the text, with or without dates, are not construed to be citations to the work of the author; e.g., *Dientamoeba fragilis* Jepps and Dobell, 1918. In this case, Jepps and Dobell are the authors of the species name *Dientamoeba fragilis* but such authorship does not entitle them to listing in Literature Cited. If used in any other bibliographic sense, an author's name would not follow, even in parenthesis, a scientific name.

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(2) Previous studies (Voge and Heyneman, 1958, J. Parasit. **44**: 249–260) have shown . . .

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—Elery R. Becker, *Editor.*

TRYPANOSOMA BALISTES N. SP. FROM *BALISTES CAPRISCUS* GMELIN, THE COMMON TRIGGERFISH, FROM THE FLORIDA KEYS*

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Trypanosomes have been reported for many years from marine fish of European waters, and a number have been described from similar hosts in Africa, Australia and New Zealand. Only a few, however, have ever been encountered in marine fish of the Americas. Kudo (1923) described the first trypanosome from marine fishes of the United States. This was found in a winter skate, *Raia ocellata* Mitchill, from Woods Hole, Massachusetts, and was considered to be identical with *T. rajae* Laveran and Mesnil, 1902. Bullock (1958) has also reported it from the common skate, *Raia erinacea* Mitchill, from southern New England.

During January and February of 1955 the writer made a general survey of the incidence of blood parasites in the marine fishes of the Florida Keys, and this paper describes a new species of trypanosome found in that material.

MATERIALS AND METHODS

Three areas of the Florida Keys were selected for collecting sites: Key West at the southern end; Marathon, approximately in the center; and Key Largo at the northern end. There were collected 1677 fish of 81 species, and a blood sample taken directly from the heart of each specimen. A thin film of each was made on a glass slide and air dried. It was then fixed in absolute methyl alcohol for one minute, washed in tap water, placed for 45 minutes in Giemsa's stain, washed in tap water, and air dried. The Giemsa stain was according to Hewitt's (1940) formula, and used as he directed. Examinations were carried out under the oil immersion objective of a compound binocular microscope.

RESULTS AND DISCUSSION

As has been described in another paper by Saunders (1958), 116 of these fish from the Florida Keys, belonging to 26 species of 13 families, were parasitized by *Haemogregarina bigemina* Laveran and Mesnil, 1901. In two common triggerfish, *Balistes capriscus* Gmelin from Key Largo, trypanosomes were found in the blood. The writer has so far examined heart blood samples from more than 5000 marine fish, and these are the first trypanosomes encountered by her. This is considered a new species as the measurements of its overall length and breadth are not the same as for any previously described marine fish trypanosome which has similar types of body ends, free flagellum and undulating membrane. It is the first record of a trypanosome from this host, and is therefore given the name of *Trypanosoma balistes* n. sp.

Trypanosoma balistes n. sp. (Fig. 1)

DIAGNOSIS

Body: Long; rather narrow; attenuated at each end, but much more so posteriorly. Polymorphism not observed.

Nucleus: Oval; stains palely; karyosome not visible; long axis transverse; occupies not more than half of body width.

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Kinetoplast: Round; red-staining; somewhat nearer nucleus than posterior end of body.
Undulating membrane: Very narrow.
Free flagellum: Between one-fourth and one-fifth of body length.
Cytoplasm: Granular; myonemes and large vacuoles absent; posterior portion stains more deeply than anterior.
Measurements (in microns): Total length, 55.5; width at level of nucleus, 3.0; body length, 43.5; posterior extremity to center of kinetoplast, 12.0; center of kinetoplast to center of nucleus, 10.0; center of nucleus to anterior extremity, 21.5; free flagellum, 12.0; width of undulating membrane, 1.0; length of nucleus, 1.2; width of nucleus, 0.6.
Host: Balistes capriscus Gmelin, the common triggerfish.
Location: Blood plasma.
Locality: Waters around Key Largo, Florida.
Holotype: Author's collection, slide No. F1553.
Paratype: Author's collection, slide No. F1554.

FIGURE 1. *Trypanosoma balistes* n. sp., from *Balistes capriscus* Gmelin, the common triggerfish.

Trypanosoma balistes n. sp. is distinguishable from previously described marine fish trypanosomes, a list of which follows:

T. soleae Laveran and Mesnil, 1901, from *Solea vulgaris,* sole. Total length, 40 microns, of which 8 microns is free flagellum.

T. scylliumi Laveran and Mesnil, 1902, from *Scyllium stellare,* dogfish. Total length 70–75 microns, of which 14 microns is free flagellum; width 5–6 microns.

T. granulosum Laveran and Mesnil, 1902, from *Anguilla vulgaris,* eel. Nucleus occupies full body width.

T. rajae Laveran and Mesnil, 1902, from *Raja punctata* and *R. mosaica,* skates. Total length, 75–80 microns, of which 20 microns is free flagellum. *T. rajae* from *R. ocellata* reported by Kudo (1923), who states nucleus fills entire body width. *T. rajae* reported from *R. batis* by Coles (1914) and from *R. erinacea* Mitchill by Bullock (1958).

T. delagei Brumpt and Lebailly, 1904, from *Blennius pholis,* blenny. Undulating membrane as wide as body; nucleus 3 times as long as wide.

T. gobii Brumpt and Lebailly, 1904, from *Gobius niger,* goby. Total length 66 microns, of which 10 microns is free flagellum; body width 5.–5.5 microns.

T. callionymi Brumpt and Lebailly, 1904, from *Callionymus dracunculus.* Total length, 70 microns, of which free flagellum is 5 microns.

T. cotti Brumpt and Lebailly, 1904, from *Cottus bubalis.* Total length 53 microns, of which 8 microns is free flagellum.

T. limandae Brumpt and Lebailly, 1904, from *Limanda platessoides,* flounder. Total length 45 microns, of which 20 microns is free flagellum; nucleus 4 times as long as wide.

T. platessae Lebailly, 1904, from *Platessa vulgaris,* European plaice. Total length 52 microns; body length 40 microns.

T. flesi Lebailly, 1904, from *Flesus vulgaris.* Width 5 microns.

T. laternae Lebailly, 1904, from *Platophrys laterna.* Free flagellum 8 microns; width 5–6 microns.

T. bothi Lebailly, 1905, (quoted by Neumann, 1909; original article not seen by author). Total length 42 microns, of which body length is 29 microns.

T. carchariasi Laveran, 1908, from *Carcharias* sp., shark. Total length 60–70 microns.

T. giganteum Neumann, 1909, from *Raja oxyrhynchus,* skate. Total length 125–130 microns, of which 25 to 30 microns is free flagellum.

T. variabile Neumann, 1090, from *Raja punctata,* skate. Polymorphic; large form, total length 90 to 100 microns, of which 25–30 microns is free flagellum; small form, total length 31 microns, of which 8 microns is free flagellum.

T. scorpaenae Neumann, 1909, from *Scorpaena ustulata.* Total length 65–70 microns; very broad undulating membrane.

T. triglae Neumann, 1909, from *Trigla corax* Bonapare, "dem Knurrhahn." Body width without undulating membrane 8 microns; undulating membrane 4 microns; nucleus 5 microns long by 4 microns wide.

T. capigobii Fantham, 1919, from *Gobius nudiceps,* dikkop. Nucleus has its long axis parallel to that of the body; "hardly any free flagellum."

T. nudigobii Fantham, 1919, from *Gobius nudiceps,* dikkop. Nucleus extends across body width; body width 6.6 to 7.5 microns.

T. sp. Fantham (1919) from *Box salpa,* bamboo fish. Body length 29 microns; body width 2 microns.

T. sp. Fantham (1919) from *Dentex argyrozona,* silverfish. Body length 20.6 microns; body width 1.3 microns; free flagellum, 2 microns.

T. pulchra Mackerras and Mackerras, 1925, from *Gilbertia semicincta* (Cuv. and Val.), half-banded sea perch, and *Parma microlepis* Gunther, white-ear. Free flagellum 7.3 microns; aflagellar end beak-shaped.

T. aulopi Mackerras and Mackerras, 1925, from *Aulopus purpurissatus* Richards, sergeant baker. "Is separated from *T. pulchra* because of its very marked pleomorphism."

T. blenniclini Fantham, 1930, from *Blennius cornutus,* blenny, and *Clinus anguillaris,* snake klipfish. No free flagellum.

T. percae var. *canadensis* Fantham, Porter and Richardson, 1942, from *Perca flavescens* (Mitchill), yellow perch, which occurs in fresh and brackish water. Body length 28.9–32.5 microns; free flagellum 5.3–6.9 microns.

T. myoxocephali Fantham, Porter and Richardson, 1942, from *Myoxocephalus octodecimspinosus* (Mitchill), long-horned sculpin. Free flagellum about 3 microns; nucleus 6.7 to 7.7 microns by 2.2 to 3 microns.

T. marplatensis Bacigalupo and de la Plaza, 1948, from *Psammotics microps,* ray. Polymorphic. Large form: nucleus perpendicular to longitudinal axis of the parasite and 4.2 by 7.2 microns. Small form: nucleus 3.6 microns in diameter.

T. heptatreti Laird, 1948, from *Heptatretus cirrhatus,* hagfish. Polymorphic. Average of 2 groups of material: free flagellum 21 to 22.6 microns; body length 60.5 to 61 microns.

T. gargantua Laird, 1951, from *Raia nasuta,* skate. No free flagellum.

T. coelorhynchi Laird, 1951, from *Coelorhynchus australis* and *Physiculus bachus.* Polymorphic. Small form: nucleus with long axis in line with that of body, free flagellum 7.5 microns. Intermediate form: total length 61.5 to 70.1 microns; nucleus 2.7 to 4 microns long by 1.9 to 3 microns wide. Large form: body width at nucleus, 4.6 microns.

T. caulopsettae Laird, 1951, from *Caulopsetta scapha,* witch, and *Rhombosolea plebia,* sand flounder. Polymorphic. Small form: reniform nucleus occupies full body width, undulating membrane same width as body. Large form: cytoplasm vacuolated, without granules; average body length 85 microns; average overall length, 102.6 microns.

T. tripterygium Laird, 1951, from *Tripterygium varium* and *T. medium,* blennies. Polymorphic. Small form: nucleus occupies full body width; body length 36.3 microns. Intermediate form: cytoplasm vacuolated; body length, 79.1 microns. Large form: overall length 100.1 microns; body width at center of nucleus 7.4 microns.

T. congiopodi Laird, 1951, from *Congiopodus leucopaecilus,* pigfish. Long axis of nucleus parallel to that of body; no free flagellum.

T. parapercis Laird, 1951, from *Parapercis colias,* blue cod. Nucleus extends full width of body; no free flagellum.

Minchin and Woodcock (1910) believe that *T. variabile* is identical with *T. rajae,* which shows marked polymorphism. A number of other marine fish trypanosomes which have been mentioned in the literature are so briefly described that their true identity cannot be told. No comparison of an unknown form with them is possible.

SUMMARY

During an examination of heart blood smears from 1677 fish of 81 species collected from the Florida Keys, trypanosomes were found in the common triggerfish, *Balistes capriscus* Gmelin. They belong to a new species and are given the name *Trypanosoma balistes*. This is the first record of any trypanosome in this fish, and is believed to be also the first record of the occurrence of a trypanosome in the blood of any marine fish from Florida waters.

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OBSERVATIONS ON THE ABILITY OF THE SNAIL *AUSTRALORBIS NIGRICANS* TO SURVIVE OUT OF WATER IN THE LABORATORY

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Studies conducted in Northeastern Brazil by Barbosa and Dobbin (1952) and Olivier and Barbosa (1955a, 1955b, 1956) have shown that the snail vectors of *Schistosoma mansoni* in that area are able to survive out of water either in the laboratory or in the natural dry habitats for different periods of time. Moreover Olivier (1956) showed that within the same species, *A. glabratus,* there were probably strain differences with respect to its ability to survive out of water. Therefore, any information concerning the survival time of the schistosome vectors from different endemic areas when kept out of water is of importance.

The vectors of *S. mansoni* in southern and central Brazil have not received the same attention as those from the northeastern part of the country. As far as is known, *Australorbis nigricans* (Spix), a planorbid vector of *Schistosoma mansoni* in certain areas in the South of Brazil, has never been tested in regard to its ability to survive out of water. Although field observations were not available, in the present paper much information was gained on the tolerance of the snails to live out of water under laboratory conditions.

MATERIAL AND METHODS

The snails used in these experiments were collected in 2 localities in the State of Rio de Janeiro, Brazil: one named Acari, near the city of Rio de Janeiro; and the other, S. Gonçalo, near Niteroi. All the snails belonging to the species *Australorbis nigricans* (Spix), the only vector of *S. mansoni* in the above mentioned areas, were collected in the field by Dr. M. Coelho in July 1957 and sent by air to the laboratory. The snails were fed with lettuce or with Standen's alginate and kept under the same conditions in well balanced aquaria. During the course of experiment 1, the snails were scattered over the wet soil contained in the large wooden box described and figured by Olivier and Barbosa (1956). The box was kept in the laboratory during the entire experiment. Water was never added, and the soil dried within a few days. All the snails were tested for viability at regular intervals by passing them through a strong beam of light. This method proved to be very satisfactory in previous experiments conducted in this laboratory (Olivier and Barbosa, 1956). The temperature of the water during this experiment ranged from 25 to 27° C, and relative humidity of the laboratory ranged from 75 to 85 percent.

In a second experiment, laboratory reared snail descendents (F4) from the wild stocks of *A. nigricans* and *A. glabratus* were used. Snails measuring from 10 to 12 mm in diameter were transferred from the water to dry clay jars about 15 cm in diameter and 7 cm deep. Snails were weighed every day for 6 days at the beginning of the experiment and at longer intervals thereafter. The temperature and humidity varied little during the experiment. Temperature ranged from 26 to 28° C and relative humidity from 65 to 75 percent. The method previously reported was used for viability.

A strain of the snail *Australorbis glabratus* from Paulista, Pernambuco, known to be very resistant to desiccation, was used in both experiments for control.

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RESULTS

Experiment 1.

In September 1957, 100 specimens of *A. nigricans* from Acari, 110 specimens of the same species from S. Gonçalo, and 100 specimens of *A. glabratus* from Paulista were scattered on the surface of the wet mud contained in the wooden box according to the method just described.

All the snails were tested for viability at 30-day intervals. Results presented in figure 1 (right) show that both strains of *A. nigricans* from Rio de Janeiro can stand desiccation but poorly when compared with the strain of *A. glabratus* from Pernambuco. Both strains of *A. nigricans* had no survivors after 90 days of desiccation, while 64 percent of the specimens of *A. glabratus* were alive at that time. Percent survival rate in *A. nigricans* was as follows: S. Gonçalo, 49.1 at 30 days and 21.8 at 60 days; Acari: 40 at 30 days and 2 at 60 days. On the other hand *A. glabratus* showed 90 percent living snails at 30 days and 73 percent at 60 days.

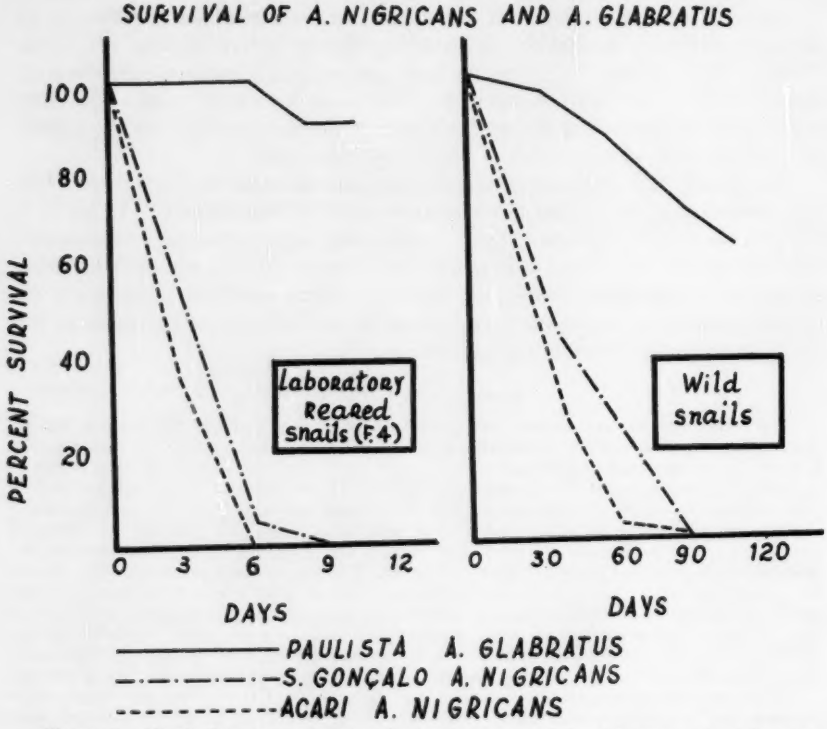

FIGURE 1. *Survival patterns of the snails* A. nigricans *and* A. glabratus *under laboratory conditions.*

Experiment 2.

One year later, in September 1958, 20 specimens of snails from each locality mentioned in experiment 1 were used. All the snails belonged to the F4 laboratory generation and had originated from the same stock collected in 1957. Results

shown in figure 1 (left) confirm a consistent difference in resistance to desiccation between *A. glabratus* of Pernambuco and the 2 strains of *A. nigricans* from southern Brazil. Moreover, snails belonging to the F4 generation were proved to be less resistant to desiccation than snails collected in the field.

The two strains of *A. nigricans* lost weight more rapidly than *A. glabratus*. The average weight loss of the 3 strains of snails was as follows. After 3 days desiccation, half of the snails from S. Gonçalo were dead and the living ones had lost 37.5 percent of their original weight. Only one snail was alive on the 6th day, and it had lost 46 percent of its weight. This snail was dead when examined 3 days later. A higher death rate occurred among the snails from Acari. After 3 days the number of surviving snails was 6, and the average weight loss was 38.8 percent. None survived 6 days of desiccation. On the other hand, the Paulista snails survived out of water for longer periods. The first 3 deaths occurred before 9 days, and after 24 days 11 snails were alive. The pattern of weight loss of these snails in percent was as follows: 3 days, 18.3; 9 days, 23.9; 15 days, 28.8; 21 days, 31.6. After 21 days the mortality was very high. Fourty-five days from the start of the experiment only the snails Nos. 3 and 11 were alive. At that time these snails had lost 33.5 (No. 3) and 41.2 (No. 11) percent of their original weight, respectively. The snail No. 11 was dead the next day and snail No. 3 died 4 days later, i.e. the fiftieth day after the start of the experiment.

DISCUSSION

While much has been published on other aspects of schistosomiasis in Brazil, very little attention has been paid to the ecology of the snail hosts except for the studies conducted in northeastern Brazil. Numerous experiments on the ability of fresh-water snails to survive out of water have been previously reported by several workers. The literature on this subject has been recently reviewed by Olivier and Barbosa (1955a) and by Olivier (1956).

The experiments described herein demonstrate clearly that 2 strains of *Australorbis nigricans,* the snail vector of schistosomiasis mansoni in the State of Rio de Janeiro, can live only for short periods out of water when compared with a resistant strain of *Australorbis glabratus* from northeastern Brazil. Deaths among the snails occurred when the weight loss attained about 40 percent, as observed by Olivier and Barbosa (1954) in *A. glabratus*. Apparently the 2 strains of *A. nigricans* did not show the same ability to retain water as *A. glabratus*. As expected, laboratory-reared snails were less resistant to desiccation than wild snails.

Results presented in this paper cannot be generalized to include all strains of *A. nigricans,* since it has been shown that there are strain differences in respect to resistance to desiccation among populations of a single species of snail. In Acari as well as in S. Gonçalo, the snails were found in permanent bodies of water. As suggested before (Olivier, 1956), strain differences were found among the snails from different habitats.

Since it was found in experiment 2 that the F4 snail progeny kept the same survival patterns when compared with their parents, it is suggested that resistance to desiccation may be regulated genetically. In that case, strain differences found to occur in *A. glabratus,* or in other snails, could be explained by adaptation of the snails to their environment with formation of geographical or microgeographical races.

SUMMARY

Experiments reported in the present paper indicate that two strains of *Australorbis nigricans* (Spix) from southern Brazil can live out of water for only short intervals of time. The snails studied were collected in Acari near the city of Rio de Janeiro and S. Gonçalo, a locality near Niteroi, State of Rio de Janeiro. A resistant strain of *A. glabratus* from northeastern Brazil was used as a control.

On the other hand, it was demonstrated that the resistant strain of *A. glabratus* kept its tolerance to live out of water in the F4 generation when compared with poorly resistant strains of *A. nigricans*.

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to Dr. Regina Martins from the "Departamento Nacional de Endemias Rurais" in the State of Rio de Janeiro, for the facilities offered for collecting the snails in the area around the city of Niteroi. Thanks are also due to Dr. M. Coelho who collected the snails in Acari and S. Gonçalo.

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A NEW SPECIES OF *AURIDISTOMUM* (TREMATODA: AURIDISTOMIDAE) FROM SNAPPING TURTLES OF GEORGIA

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Twenty-eight specimens of a medium-sized trematode were recovered from the small intestines of 2 snapping turtles, *Chelydra serpentina serpentina* Stejneger, collected in Bulloch County, Georgia, in 1958. It was determined that the worms belonged to the genus *Auridistomum* Stafford, 1905, and that they closely resemble *A. chelydrae* (Stafford, 1900). In the author's opinion, however, they differed in enough respects to constitute a new species.

The living worms were firmly embedded in the mucosa of the small intestine, and it was with difficulty that they were extracted in their entirety. The following description is based on specimens fixed in hot Bouin's fluid and stained with acidified Harris' alum hematoxylin. Sectioned material, at 7 and 15 microns, was stained with Mallory's triple stain. The following measurements, stated in millimeters, were taken from 10 specimens mounted in toto.

Auridistomum georgiense n. sp.

Diagnosis: Elongate distome with constriction at middle of body. Spination pronounced anteriorly, diminishing posteriorly. Body 4.24 (3.24–4.99) long by 0.876 (0.428–1.103) wide at level of testes. Subterminal oral sucker 0.306 (0.226–0.360) in diameter provided with pair of lateral projections. Acetabulum 0.213 (0.188–0.240) in length by 0.218 (0.158–0.248) in width. Pharynx slightly wider than long, measuring 0.202 (0.180–0.233) in length by 0.212 (0.188–0.240) in width. Esophagus lacking in all specimens. Ceca extending entire length of body. Testes, slightly oblique, lobed in outline, just posterior to body constriction; anterior testis, 0.309 (0.255–0.375) long by 0.298 (0.225–0.345) wide; posterior testis, 0.301 (0.225–0.375) long by 0.307 (0.248–0.375) wide. Long, claviform cirrus sac extending from anterior margin of acetabulum to genital pore. Large, coiled seminal vesicle in posterior portion of sac. Ejaculatory duct and anterior portion of seminal vesicle surrounded by large cells of prostate gland. Genital pore midventral at bifurcation of intestine (fig. 2, GP). Elliptical ovary measuring 0.243 (0.210–0.315) by 0.274 (0.240–0.310) located immediately posterior to and dextral of acetabulum.

Oviduct joined by prominent seminal receptacle posterior to ovary. Laurer's canal reduced or lacking in specimens examined. Egg-filled uterus extending from anterior margin of anterior testis to genital pore; lateral folds of uterus extend extra- and intercecally. Vitellaria extensive and continuous in posterior portion of body to level of anterior testis from which point they continue as two lateral bands to level of ovary (figs. 1 and 5). Eggs elliptical, 0.032 (0.031–0.033) by 0.014 (0.013–0.016). Excretory bladder long, extending nearly to acetabulum where it forks into several branches. Excretory pore terminal. Protuberance on mid-dorsal surface at level of ovary and seminal receptacle (figs. 3 and 4, P).

Host: Chelydra serpentina serpentina Stejneger.

Locality: Bulloch County, Georgia.

Type Specimens: U. S. N. M. Helminth. Coll. No. 39016.

DISCUSSION

In North America, the only member of the genus *Auridistomum* Stafford, 1905, is *A. chelydrae* described by Stafford (1900, 1905) from snapping turtles found in Canada. In his descriptions, Stafford failed to specify many of the dimensions of the parasite. This prompted Williams (1953) to redescribe the worm from material

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collected from the type hosts of Oklahoma. Yamaguti (1958) has reduced *Pterygomaschalos attenuatus* Stunkard, 1924, and *Tetrapapillatrema concavocorpa* Ralph, 1938, to synonomy with *A. chelydrae*. The following comparisons, therefore, are made with *A. chelydrae* and those forms now included in the genus as set forth by Yamaguti. Further comparative studies were facilitated by the acquisition of material from the type hosts on deposit with the U. S. National Museum (Helminth. Coll. No. 45702).

Auridistomum georgiense can be distinguished from all previously described forms of *A. chelydrae* by the lobed testes, its greater overall size, and the nature of Laurer's canal.

The testes are regular in outline in the forms described by Stafford (1900, 1905), Stunkard (1923, 1924), Ralph (1938), Williams (1953), and in the material from the type hosts. The present specimens are much larger than those described by Stunkard and Williams. However this criterion may not prove reliable because the few dimensions determined by Stafford and those presented by Ralph fit well into the range of those established by this author.

The greatest distinguishing characteristic that separated *A. georgiense* from its closest related species is the lack of a prominent Laurer's canal. Stafford (1905) described this structure as opening ". . . in the mid-dorsal line by a conspicuous thick-walled opening." Williams (1953) described Laurer's canal as being ". . . thick-walled, in fork of excretory ducts." Further examination of specimens from the type host revealed details similar to those described by the two authors (e. g., the thick-walled Laurer's canal opening to the exterior, on the mid-dorsal surface, by means of a prominent elliptical aperture). Examinations of whole mounts and sectioned material of *A. georgiense* failed to reveal such structures. All the specimens in the author's collection did possess, on the mid-dorsal surface, a muscular protuberance at the level of the ovary and seminal receptacle. Sections through this protuberance revealed a well-developed layer of longitudinal muscle with a noncellular "plate" imposed upon it. No clue as to its function was apparent. A possible explanation is that it represents a vestige of the muscular opening to Laurer's canal, and it may possibly be indicative of the disappearance of the canal in this particular species.

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Explanation of Plate I

(All figures drawn with the aid of camera lucida)

Figure 1. *Auridistomum georgiense* n. sp. Dorsal view of specimen.

Figure 2. *Auridistomum georgiense* n. sp. Section through the genital pore, showing egg in the pore.

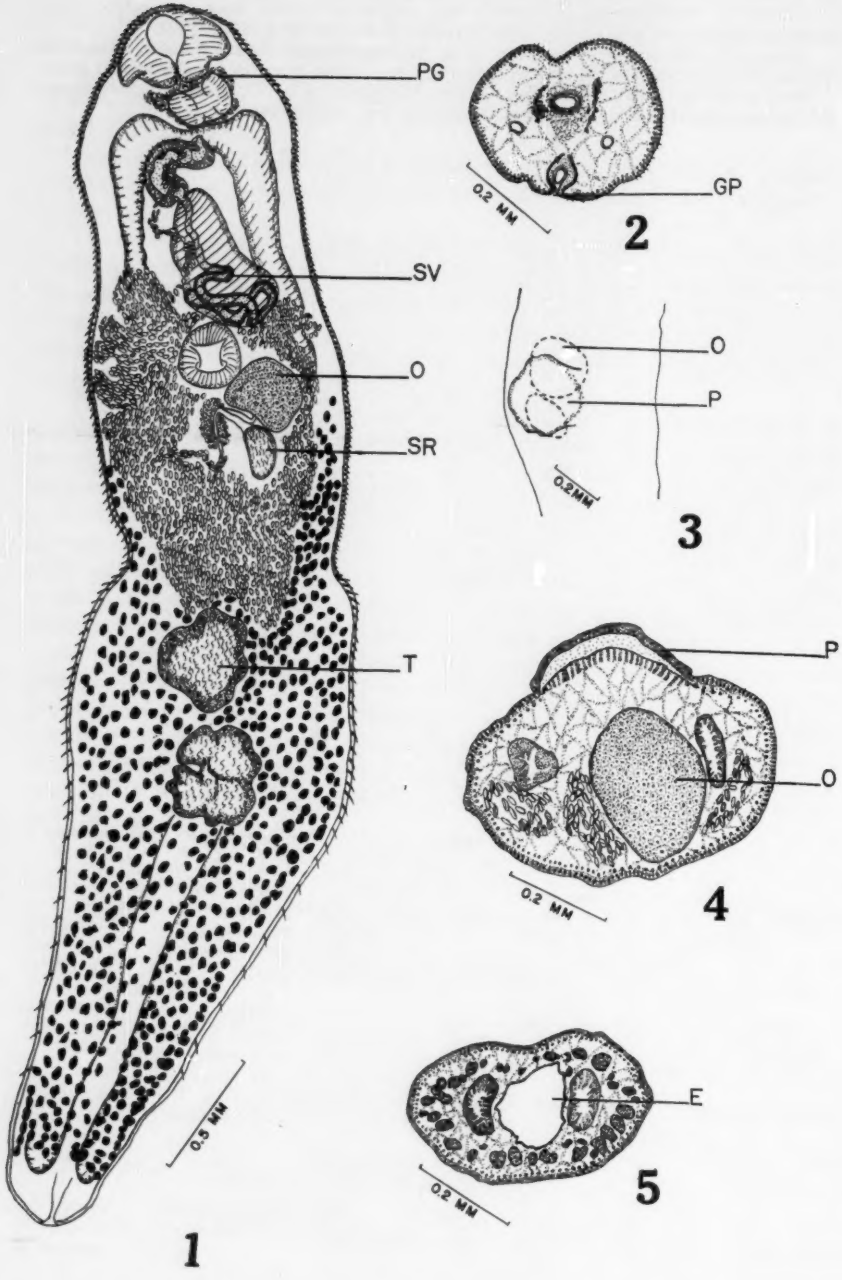
FIGURE 3. *Auridistomum georgiense* n. sp. Dorsolateral view of portion of specimen showing protuberance in relation to ovary and seminal receptacle.

FIGURE 4. *Auridistomum georgiense* n. sp. Section through the ovary and protuberance of specimen, showing the relative size of the muscular layers and noncellular "plate."

FIGURE 5. *Auridistomum georgiense* n. sp. Section through posterior region of specimen, showing the distribution of the vitellaria and the nature of the excretory bladder at that level.

Key to abbreviations: E, excretory bladder; GP, genital pore; O, ovary; P, protuberance; PG, peripharyngeal glands; SR, seminal receptacle; SV, seminal vesicle; T, testis.

PLATE I



THE LARVAL DEVELOPMENT OF *STELLANTCHASMUS FALCATUS* (TREMATODA: HETEROPHYIDAE) IN THE FIRST INTERMEDIATE HOST

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There is little known of early developmental stages in the heterophyids. In the description of the life cycle of *Centrocestus formosanus,* Nishigori (1924) did not describe a sporocyst stage. Stunkard (1930) reported finding no early sporocyst stages of *Cryptocotyle lingua.* Cable (1934) did not find sporocysts of *C. lingua* in examinations of sections of the digestive tract of *Littorina littorea.* Wootton (1957) examined hundreds of snails but did not find sporocysts stages of *Cryptocotyle concavum.* Martin (1958) briefly described young sporocysts of *C. formosanus* as solid, with older ones having a core of cells at least partially separated from the wall.

So far as the writer is aware, there has been no description of the mother sporocyst in the heterophyids. Furthermore, the type of life cycle in this heterophyid in which a mother and a daughter sporocyst are followed by redial generations constitutes a form of development unknown in other groups of trematodes. The present paper describes the mother sporocyst and other larval stages of *Stellantchasmus falcatus* in the first intermediate host.

MATERIALS AND METHODS

Adult *S. falcatus* worms were obtained from a kitten previously fed infected mullet, *Mugil cephalus.* Worms were collected in a modified Baermann apparatus; other heterophyids, when present, were removed. Worms were crushed, and juvenile *Tarebia granifera mauiensis* (Lea) and *Melanoides newcombi* (Lea) were allowed to feed on the crushed material which contained the eggs of the flukes. After 24 hours, the snails were removed to another dish and later examined at various intervals.

In the examination for larval stages, the snail shell was gently crushed and the broken parts carefully removed with sharp dissecting needles under a dissecting microscope. The snail was placed on a watch glass in tap water and examined grossly under the dissecting microscope and low and high powers of the compound microscope. The head region was then removed and the body examined under a coverslip. Sporocysts were studied unstained or stained intravitally with neutral red.

All snails used in the experimental infections were laboratory reared and were fed alginate food (Lee and Lewert, 1956). They were raised and maintained indoors in glass dishes in which the water temperature remained fairly constant at around 78° F.

The snail hosts, *T. granifera* and *M. newcombi,* were formerly reported by the writer (Noda, 1958) by their synonyms, *Melania mauiensis* and *M. indefinata,* respectively. The larval stages described herein were obtained only from infections in *T. granifera.*

All measurements are expressed in millimeters.

OBSERVATIONS

Eggs and miracidia. Eggs were first observed in cat feces on the 8th day following the feeding of infected *Mugil cephalus.* The eggs are oval, 0.019 to 0.025 in length, av. 0.022, and 0.012 to 0.016 wide, av. 0.013. They are golden brown and

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details of the miracidium were obscured. The eggs are apparently infective when oviposited.

Mother sporocyst. At intervals following experimental infection all tissues of the snail, especially the digestive tract (esophagus and intestine), were carefully examined for early larval stages. The first definite observation of a mother sporocyst was 14 days following experimental infection. At this period, each mother sporocyst when viewed superficially under low power of the compound microscope appeared as a small ovoidal body, about 2.0 long, with irregular and dark pigmented surface. From each of several snails examined, from 2 to 10 of these bodies were noted, usually about midway along the length of the intestine. When observed under coverslip preparations, each body was found to be composed of several ovoidal compartments, each about 0.088 to 0.107 in diameter and enclosing a single germinal cell which gives rise to the daughter sporocyst.

The compartments which make up the mother sporocyst appear to be similar to those of *Plagiorchis muris* as described by Cort and Olivier (1943) and Cort and Ameel (1944). The compartment wall is thin (about 0.003 thick) and contains oval nuclei, approximately 0.001 to 0.002 in length.

Between the 19th and 39th days the single germinal cell within each compartment undergoes unequal cleavage followed by the production of many oval cells, 0.009–0.014 in diameter (fig. 3). During this period the cells became enclosed by a membrane which has been observed to separate from the compartment wall and which condenses into the shape of an oval body within the compartment. This membrane, whose origin is not clear, becomes the inner wall of the daughter sporocyst. (The outer wall of the daughter sporocyst is the original wall of the compartment.) At first this stage is oval but eventually becomes elongated (figs. 4 and 5). With further growth and elongation of each daughter sporocyst, as in figures 6 to 8, the compartment-like appearance of the mother sporocyst is lost.

Although the snails were infected at the same time, considerable difference was noted in growth rate of the larvae in the various snails examined. In one snail examined 39 days after experimental infection, the mother sporocyst was still in early cleavage; another snail examined on the same day had elongate daughter sporocysts. The difference in growth rate has been noted in all stages of larval development.

Daughter sporocyst. In the early development of the daughter sporocyst, one end of the growing embryo has many compact cells, and the remainder of the embryo contains scattered individual cells. The daughter sporocyst often appears reticulated (fig. 10) with a network of thin, connecting cytoplasmic filaments. In some instances, cells appear to be suspended by this thin filament.

The cellular mass within the elongating daughter sporocyst shows no constant shape. At 29 days, some of the daughter sporocysts have elongated to as much as 0.4. At 36 days, one of the daughter sporocysts measured approximately 2 mm. With further growth, the cellular mass resembles a chain (fig. 9) and there is further separation from the wall. In a few instances, prior to "chain formation" there was observed in the periphery of the mass a group of 15 to 25 cells 0.055 in diameter. This, presumably, is the germ ball of the mother redia.

The outer wall enclosing the daughter sporocyst varies considerably in thickness. It may be as thin as 0.003 or as thick as 0.043. It appears to be quite vacuolated in the thicker portions (fig. 10). The thickness does not appear to be corre-

lated with the number of cells which make up the wall, because some thick areas have relatively few, sparsely-distributed cells, whereas the thinner areas are often 2 or 3 cells thick.

A daughter sporocyst has not been isolated in its entirety. The longest thread measured approximately 12 mm. These thread-like sporocysts are, in part, in intimate contact with the host tissue, or tangled with other sporocysts. At this stage the intestinal area of the snail is very distended, appears grey, and when dissected the mass of threads is found to completely envelop the feces-packed intestine. The daughter sporocysts when isolated in water showed no motility.

Mother redia. In some daughter sporocysts at 55 days following infection, the "chain-like" appearance is evident and rediae-shaped bodies are recognized. In isolated cases, the pharynx is discernible at this time. In later development, the pharynx is very active and the saccular gut, which is approximately one-half to three-fourths the length of the redia, is clearly observed. Within the sporocyst, rediae with pharynx and gut vary in size, ranging in length from 0.09 to 0.22, av. 0.14, and in width from 0.050 to 0.086, av. 0.062.

In the immature mother redia there are many nuclei scattered in the connective tissue in the region of the future body cavity, as well as around the gut and pharynx. Later, there is a concentration of germinal cells in the posterior region and a few single cells in the body cavity. With further development the germ balls fill the anterior end of the body cavity. The anterior redial embryos are more advanced in development than those posterior, so that there is a graduation of redial development. This anterior-posterior gradient of development has been reported by Cable (1934) in *Cryptocotyle lingua,* and Ameel et al (1950) in *Euryhelmis monorchis.*

The first mother rediae found outside of the daughter sporocyst were located in the lymph space around the stomach of a snail 68 days following infection. There were 9 rediae in this area, and none was present in the digestive gland. At 88 days, 33 mother rediae had migrated into the digestive gland. Measurements of 12 rediae are: length, 0.16–0.30, av. 0.22; width, 0.050–0.067, av. 0.056; transverse diameter of pharynx, 0.023–0.027, av. 0.025. The gut was approximately one-half of the body length.

That larval development varies from snail to snail was particularly evident in one snail examined 134 days after infection. In this snail, there were no rediae in the digestive gland or in the lymph space around the stomach. The intestinal area was very distended, and examination of daughter sporocysts showed many rediae comparable in size and development to those found earlier in migration or in the digestive gland.

Daughter redia and cercaria. At 97 days after infection the digestive gland of the snails examined contained many mother rediae and young daughter rediae. Similar to the development of daughter rediae, cercariae develop from germinal cells located in the posterior region of the redia. In mature daughter redia, 1 to 3 cercariae with pharynx and eye spots are located in the anterior region of the body cavity. The tail, at this time, lacks fin folds. Between the cercariae and germinal cells are a number of germ balls. Mature daughter rediae with developing cercariae measured as follows: length, 0.33–0.68, av. 0.49; width, 0.09–0.15, av. 0.12; transverse diameter of pharynx, 0.025–0.027, av. 0.026.

The cercaria is pleurolophocercous with eye spots. Morphological features have been described by Martin (1958). On leaving the daughter redia, they complete

their development in the digestive gland. Cercarial emergence was first noted 123 days after infection.

Young uninfected mullets, about 1 to 1.5 inches in length, were exposed to cercariae which had emerged from experimentally infected snails. Sixteen days after infection, these mullets were fed to laboratory reared rats. Adults of *S. falcatus* were recovered from the intestine of autopsied rats.

DISCUSSION

Although several life history studies have been previously reported by other investigators of the heterophyids, little has been reported of the early larval stages in the first intermediate host. Martin (1958) found slender, elongate sporocysts of *C. formosanus* in the mantle tissue near the gills in some snails. If the sporocyst development of *C. formosanus* and *S. falcatus* found in this study is typical of the heterophyids, then examinations of the intestinal area, rather than the digestive gland, would expose the early larval stages of heterophyids. Furthermore, as was found in this investigation, the mother sporocyst is a rather short-lived stage; i.e., all vestiges of the mother sporocyst, except the outer wall of the daughter sporocyst, disappear with the growth of the daughters.

The earliest mother sporocyst observed was a small body with several compartments each containing a germinal cell. It appears that the stage observed is in one respect comparable to the description of the early mother sporocyst of *P. muris* by Cort and Ameel (1944); viz., the mother sporocyst is composed of several compartments wherein following cleavage single or many cells are observed.

Observations showed that the single germinal cell undergoes repeated cleavage to produce the daughter sporocyst embryo. With further development, the daughter sporocyst elongates into a long thread. There is no migration of the daughter sporocyst. It is nonmotile, and although its width may vary throughout its length, it is a continuous thread without branching.

Although no attempt was made to determine the number of compartments in the mother sporocyst, the impression obtained is that they are relatively few in number. This would indicate that either the number of germinal cells in the miracidium is small, or that in the metamorphosis of miracidium into mother sporocyst, germinal multiplication, if it occurs, is quite limited, since there are only 300 to 500 daughters, as reported for *P. muris* by Cort and Ameel (1944).

In *S. falcatus,* daughter sporocysts give rise to mother rediae. In immature daughters, there is present a group of germinal cells in one end of the sporocyst in addition to the single cells in the body cavity. Germ masses were not observed. In later stages, germ balls were observed. The fate of the single germinal cells up to the formation of mother redial embryo cannot be stated with certainty.

In the digenetic trematodes, it is the usual concept that both daughter sporocysts and redial generations do not occur in the same life cycle. The finding of daughter sporocysts and redial generations in the life cycle of *S. falcatus* adds another variation in the life cycles of digenetic trematodes.

Reports of investigators on trematode life cycles often indicate maturity and emergence of cercariae in a month or 6 weeks. In view of this, the length of time from date of infection to cercarial emergence in this study was surprising. Cort

et al (1954) suggested that the increase in size of the sporocysts in large snails is greater than in laboratory reared juveniles due to more space and availability of food.

SUMMARY

The development of the larval stages of the heterophyid, *Stellantchasmus falcatus,* in the molluscan host has been studied, and the stages in the life cycle found to consist of mother and daughter sporocysts, mother and daughter rediae, and cercarial stages. The occurrence of both daughter sporocysts and redial generations in the same life cycle is a phenomenon not previously reported in the life cycles of digenetic trematodes.

The earliest mother sporocyst was found near the intestine, midway between the stomach and anus. It was composed of several compartments, each containing a germinal cell. Repeated cleavage of these germ cells resulted, directly, in the daughter sporocysts. The daughter sporocyst developed in place, that is, without migrating to a new locus. The mother rediae, which develop in the daughter sporocyst, do migrate to the digestive gland and give rise to daughter rediae. Cercariae develop inside daughter rediae whence emergence was first noted 123 days after snails were exposed to fluke eggs.

ACKNOWLEDGMENT

The author wishes to thank Dr. J. E. Alicata for suggesting the study and for advice during the course of investigation.

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PLATE I

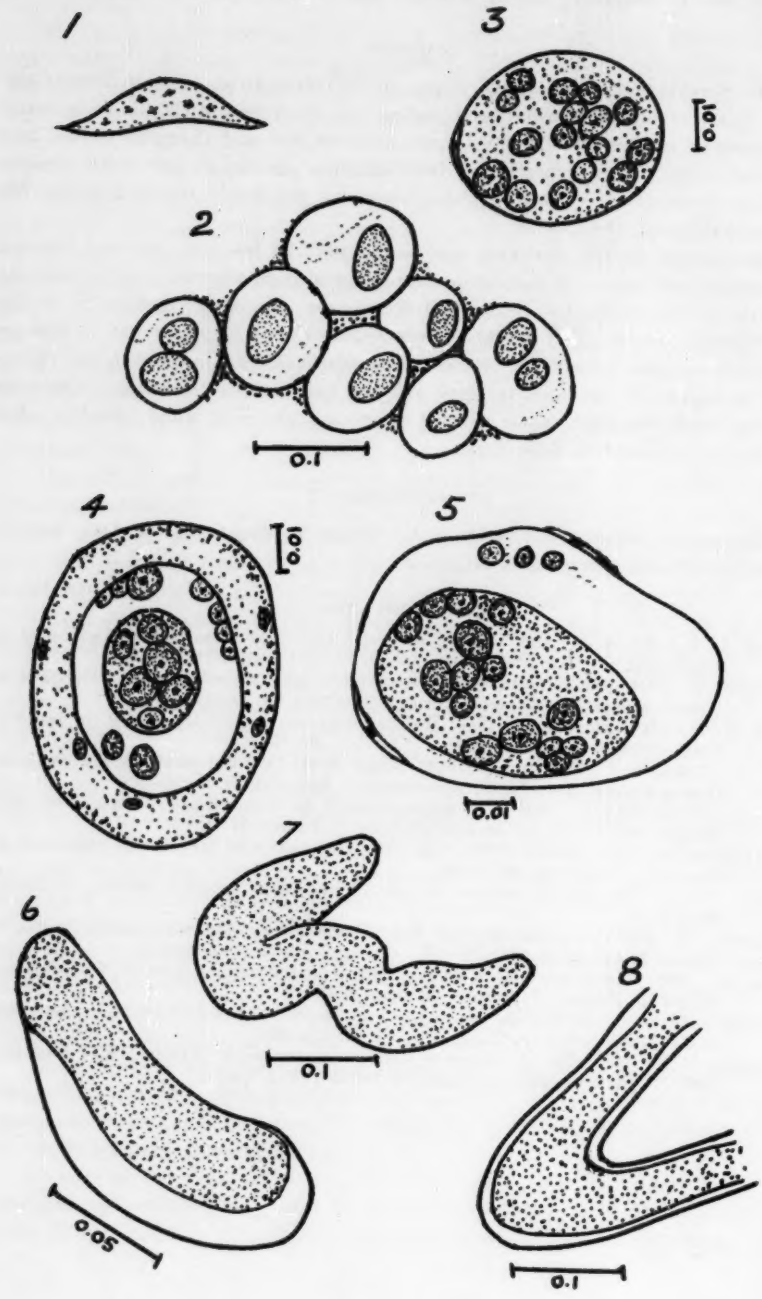
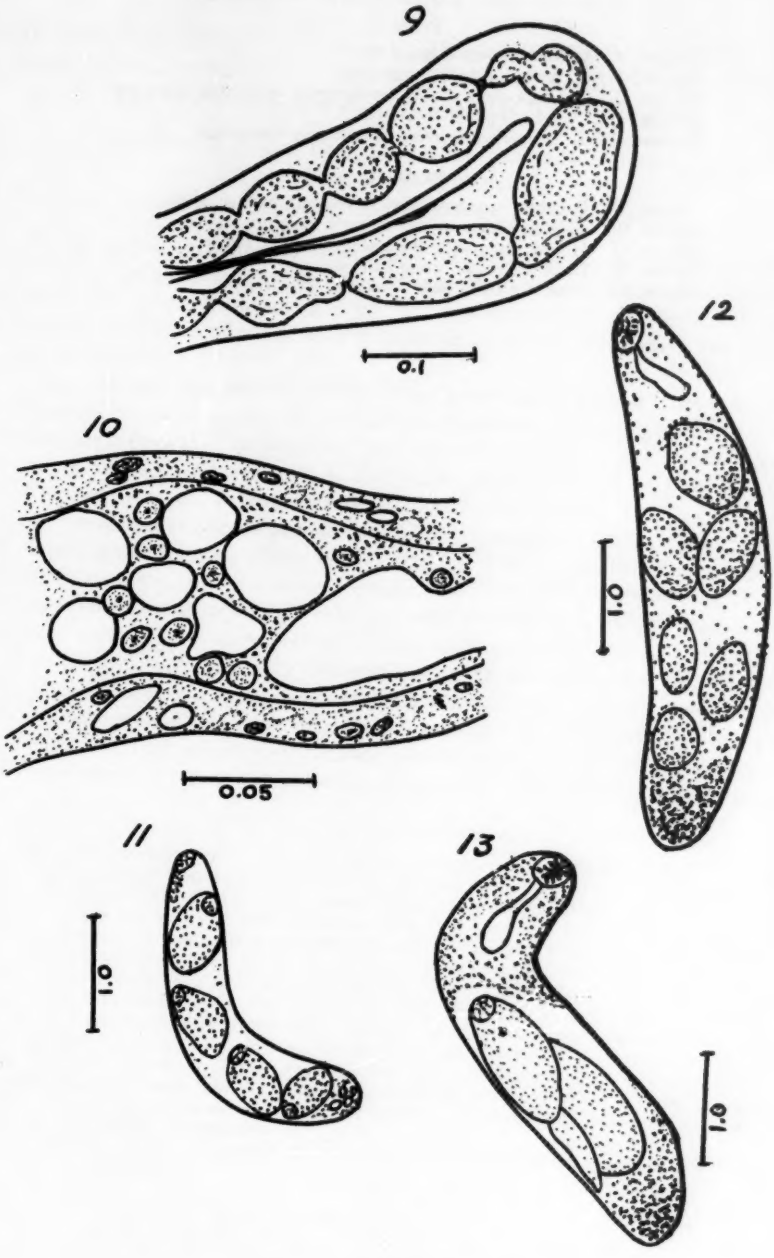


PLATE II



Explanation of Plates

(Figs. 2–13 drawn with the aid of a camera lucida)

Plate I

Figure 1. Mother sporocyst, ca 2.0 mm.
Figure 2. Portion of a mother sporocyst.
Figure 3. Cleavage stage in one compartment of mother sporocyst.
Figures 4, 5. Early daughter sporocysts.
Figures 6, 7, 8. Daughter sporocysts, beginning elongation.

Plate II

Figure 9. Daughter sporocyst with developing mother rediae.
Figure 10. Vacuolated appearance of early daughter sporocyst.
Figure 11. Mother redia with developing daughter rediae.
Figure 12. Daughter redia, "germ ball" stage.
Figure 13. Daughter redia with developing cercariae.

THE PATHOGENESIS OF TRICHOSTRONGYLOID PARASITES. III. SOME PHYSIOLOGICAL OBSERVATIONS IN LAMBS SUFFERING FROM ACUTE PARASITIC GASTROENTERITIS*

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During recent years, increased emphasis has been placed on the physiological and biochemical alterations which occur in animals and man during the course of nematode infections. A considerable part of this work has been directed toward the mechanism of anemias which develop in lambs infected with members of the family Trichostrongylidae.

It has been well established (Fourie, 1931; Andrews, 1942) that when members of the genus Haemonchus are the predominant parasites, the anemia produced is of a hemorrhagic nature. The peripheral blood picture, however, may be quite varied depending on whether the disease is complicated by a deficiency of iron or some other nutrient essential to hemopoiesis (Whitlock, 1949; 1950).

The mechanism of anemia produced by other genera of this family is not so clear cut. Most texts (Lapage, 1956a; 1956b) attribute it to the bloodsucking propensities of the parasite. Other writers (Schalm, 1956) have found indications of decreased erythropoiesis, while still others (Baker and Douglas, 1957b) have noted a reduced survival time of red cells within the vascular system.

The present paper presents observations on hematological and physiological alterations in sheep which had acquired clinical infections of several genera of the family Trichostrongylidae while on irrigated pasture. It is believed that the data, while not permitting definitive conclusions concerning the mechanism of the disease, will contribute to a better understanding on which to base future investigations.

MATERIALS AND METHODS

Ten lambs showing varying degrees of clinical symptoms were obtained on July 10, 1958, from a ranch in Placer County, California. One additional lamb from the same ranch which had been in the University of California Veterinary Clinic was obtained. These lambs had been on irrigated pasture and were still with their dams up to that time. On the basis of an autopsy of 2 additional lambs, as well as fecal examinations, it was concluded that *Haemonchus contortus* was principally responsible for the severe anemia present in several of them, although rather large numbers of *Ostertagia* sp., *Trichostrongylus* sp., and *Nematodirus* sp. were present. Four of the lambs with acute anemia died by July 28, 1958, 18 days after arrival at the University. On July 18, 6 ewe lambs were treated with 25 g of N. F. commercial grade phenothiazine. This group included 1 lamb (549) from the 4 mentioned above. Beginning on July 22, 1958, 2 ewe lambs (350, 596) were given 200 mg $FeSO_4$ per os daily. Chromium 51 red cell survival times were determined in 2 lambs with No. 453 being injected on July 16, 1958, and No. 547 on August 1, 1958. At autopsy, livers were obtained for chemical analysis and the abomasums and small intestines for nematode enumeration.

The methods used for studying the peripheral blood hemograms were essentially those developed by Wintrobe.

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The plasma iron (manifest iron-binding capacity, MIBC), latent iron-binding capacity (LIBC), total iron-binding capacity (TIBC) and saturation percent were determined as previously described (Baker and Douglas, 1957a). It should be noted that in item 9 of the procedure for plasma iron presented in that publication the concentration of ammonium hydroxide was given as 3.5 M, rather than the correct molarity of 7.0.

Plasma copper values were determined by the method of Gubler et al (1952). Liver hemoglobin was determined by the technique of Greenberg and Erickson (1944). The methods used for determining total liver copper and iron and those of Chase et al (1952) with the following modifications. Two grams of tissue were digested in 3 ml of nitric acid and then brought to a volume of 25 ml. A 5 ml aliquot of this fraction was then placed in a 100 ml Kjeldahl flask along with 2 ml of nitric acid and 1 ml of perchloric acid. This mixture was then wet ashed and diluted to a volume of 25 ml. For the copper determination, 5 ml of this solution was added to 0.25 ml of a 2 to 1 dilution of ammonium hydroxide with water and the procedure continued as presented by Chase et al. For the total iron determination, 3 ml of the same wet ashed material was added to 0.29 ml of 7 M ammonium hydroxide and brought to volume (5 ml) with redistilled water. To this solution was added 5 ml of composite reagent (Baker and Douglas, 1957a), and the procedure was continued as for plasma iron determination. Liver storage iron was estimated by subtracting hemoglobin iron from total iron.

In order to obtain an estimate of the normal excretion of chromium 51, two lambs from the University flock which were free of parasites, as evidenced by fecal examination, were selected for trial. In these animals, however, the procedure differed slightly in that the red cell labeling process was stopped by the addition of 50 mg of ascorbic acid solution and the cells were not washed prior to reinjection.

Total parasite counts and species composition of the nematode population were performed as previously reported (Douglas, Baker, and Longhurst, 1956).

RESULTS

Parasitological

Data pertaining to total nematode counts and species composition of the worm populations are presented in table I. At autopsy, only 3 lambs, 551, 550A, and 453,

TABLE I. *Total worm counts and species composition of worm populations at autopsy.* (M, male ; F, female)

Species	7/16 551 M*	7/17 550A F	7/24 549 F**	7/28 453 M	8/12 547 M	9/8 350 F	7/8 450 M	9/11 546 F	9/11 550B F	9/25 548 F	9/25 476 F
Abomasum											
O. circumcincta	12,075	12,040	5,640	12,919	5,549	526	527	2,260	340	572	1,120
O. trifurcata	1,825	—	—	—	—	174	—	—	—	78	—
T. axei	3,350	3,200	508	591	3,121	—	93	—	30	—	—
T. colubriformis	—	—	—	—	—	80	—	—	30	—	—
H. contortus	5,750	6,220	—	1,775	—	—	—	—	—	—	—
Totals	23,000	21,460	6,148	15,285	8,670	780	620	2,260	400	650	1,120
Small intestine											
T. colubriformis	16,614	3,003	5,573	6,323	8,900	1,612	10,584	1,621	2,175	360	1,625
T. vitrinus	—	501	2,018	1,969	—	403	—	288	445	290	—
N. spathiger	—	328	—	2,073	523	355	588	628	—	—	—
N. filicollis	1,846	330	1,514	—	—	—	—	—	—	290	85
S. papillosus	—	—	—	—	—	—	—	313	—	60	—
C. curticei	—	—	—	—	1,047	—	—	—	—	—	—
C. punctata	—	218	505	—	—	—	588	—	—	—	—
Totals	18,460	4,380	9,610	10,365	10,470	2,370	11,760	2,850	2,620	1,000	1,710

* Lambs 551, 550A, 549, and 453 died early in the study.
** Lambs 549, 350, 546, 550B, 598, and 476 received phenothiazine on July 18, 1958.

were found to be harboring *H. contortus*. The explanation for this is found in the fact that lambs 549 and 476, which demonstrated marked anemia and fecal egg counts of 37,500 and 19,250, respectively, were treated with phenothiazine prior to autopsy. One additional lamb, 546, while not exhibiting marked anemia, had an egg count of 16,250 per g which was indicative of clinical infection with *H. contortus*. The remaining 5 lambs had fecal counts ranging from 100 to 6,550 per g. In all animals treated, phenothiazine significantly reduced the egg counts, presumably due to its relatively high anthelmintic efficiency against *H. contortus*.

Peripheral Blood Studies

With the exception of the 4 animals which died early in the study, only one, 476, showed a marked anemia. Table II presents the hematological data concerning 3

TABLE II. *Haematological data on four anemic lambs.*

Animal	Sex	Sample date	Hb g/100 ml	PCV (pct.)	RBC cells/ $mm^3 \times 10^6$	Ret. (pct.)	Nucleated RBC (pct.)*	MCH uug	MCV u^3	MCHC (pct.)*
550A	F	15 July	1.6	7.0	1.65	1.0	1.0	9.7	42	23.0
549	F	15 July	4.8	15.0	5.51	0.1	0	8.7	27	32.0
		23 July	4.4	14.0	5.07	0.1	0	8.7	28	31.5
453	M	16 July	4.3	15.0	3.96	0.6	1.0	10.9	38	29.0
		18 July	3.5	12.0	3.78	0.1	0	9.3	32	29.0
		25 July	2.2	7.0	2.5	0.1	0	8.8	28	31.0
476	F	21 July	5.2	17.0	6.03	0.1	0	8.6	28	30.0
		23 July	4.9	16.0	5.2	0.1	0	9.4	31	30.5
		5 August	7.5	26.0	8.7	0.1	0	8.6	30	29.0
		8 August	7.2	25.0	8.1	0.1	0	8.9	31	29.0
		19 August	8.2	27.0	8.78	0	0	9.3	31	30.0
		29 August	8.8	28.0	9.8	0	0	9.0	29	31.0
		29 Sept.	10.0	33.0	10.73	0	0	9.3	30.5	33.0

Ret., reticulocytes; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin.
* Nucleated RBC/100 W. B. C.

which died early and animal No. 476.

On the basis of normal values from 12 control lambs, in which the mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were found to average 32.9 cubic microns (30.4–36.0) and 32.7 percent (27.8–36.3), respectively, the following deductions can be made from table II. (1) Lamb 550A exhibited a macrocytic hypochromic anemia. (2) Lamb 549 demonstrated a microcytic normochromic anemia. (3) Lamb 453 showed a macrocytic, and perhaps slightly hypochromic anemia on July 16; and in a period of 9 days, progressed to a microcytic normochromic anemia. (4) Lamb 476 exhibited a marked anemia which tended to be of a microcytic and hypochromic type.

During the anemic stage all of these 4 animals showed anisocytosis, poikilocytosis, moderate degrees of hypochromasia, and excessive rouleau formation. In general, all of the foregoing observations are commensurate with hemorrhagic anemia and subsequent iron deficiency.

None of the remaining lambs exhibited marked anemia, although 2 ewe lambs and 1 wether lamb had hemoglobin levels of 9 to 10 g per 100 ml prior to August 1. Subsequent to this, hemoglobin levels in the 2 ewe lambs were above 10 g per 100 ml. Hemoglobin values in the wether lamb did not reach this level until August 27. The packed cell volumes, red blood cell counts, mean corpuscular volumes, and mean corpuscular hemoglobin concentrations were essentially normal, although prior to August 5 there was a slight decrease in the mean corpuscular volume and packed cell volume of the 3 animals mentioned above.

Plasma Copper Levels

Normal values for plasma copper levels in this laboratory have ranged from 100–160 micrograms per 100 ml during summer months. In a limited number of animals examined during the months of September, October, and November, these values in the same lambs progressively decreased to as low as 58 micrograms per 100 ml.

In the lambs which died by July 28, 1958, the plasma copper values ranged from 76 to 197 micrograms per 100 ml, with a mean of 117 micrograms per 100 ml. There

was no correlation between the degree of anemia and the plasma copper level.

In the remaining lambs the plasma copper values in July ranged from 83–144, with a mean of 121 micrograms per 100 ml; in August the range was 63–120, with a mean of 90 micrograms per 100 ml; and in September the range was 63–106, with a mean of 80 micrograms per 100 ml.

It is noted that neither the range nor the mean of plasma copper values of the severely anemic lambs differs significantly from that of the less affected animals during July. In both groups, the lower values are below previously obtained normal values; however, it is doubtful that this can be considered of significance at the present time. After July 31, there was a progressive decrease of plasma copper levels in surviving lambs. The reason for this decrease is not known. In view of the decrease observed in normal lambs, these changes may well represent a normal physiological cycle.

Plasma Iron Values

Table III presents the data on plasma iron values for the 4 lambs which died early in the study.

TABLE III. *Plasma iron values of lambs dying early in the study.*

Animal	Sample date	MIBC ug/100 ml	LIBC ug/100 ml	TIBC ug/100 ml	Percent saturated
550A	7/15/58	55	376	431	12.8
551	7/15/58	85	265	350	24.3
549	7/15/58	40	257	297	13.5
	7/23/58	85	288	373	22.8
453	7/16/58	55	285	340	16.2
	7/25/58	63	—	—	—
Average		64	294	358	17.9
Normal** wether lambs		131 ± 7	196 ± 14	327 ± 11	40 ± 3
Normal ewe lambs		135 ± 4	204 ± 8	339 ± 6	39 ± 3

MIBC, manifest iron binding capacity or actual plasma iron; LIBC, unsaturated iron binding capacity (measurement of unbound transferrin); TIBC, total iron binding capacity (MIBC and LIBC).
* Sample consisted of heart blood. ** (Baker and Douglas, 1957a.)

It is noted that all 4 animals with acute anemia showed a markedly reduced MIBC and percent saturation of transferrin. All other lambs with the exception of 476 showed normal plasma iron components. The latter animal was markedly anemic in July, but had lost the nematodes of the genus *Haemonchus,* presumably due to an immunological response. During recovery this lamb showed a variable, but generally low, MIBC and TIBC.

Tissue Copper and Iron Stores

Normal values for liver copper have been found to be quite variable in this laboratory. Liver copper values as low as 4 mg per 100 g (fresh wt) and as high as 25 mg per 100 g have been found in animals which were in good health and showing normal blood values.

Values for liver copper in lambs examined in this study ranged from 11.9 to 20, with a mean of 16.9 mg per 100 g, and 10 to 27.1, with a mean of 17.0 mg per 100 g for the lambs which died with severe anemia and the remaining animals, respectively. Thus, it can be concluded that liver copper stores were not depleted in any of the animals.

Normal values for liver iron have also been found to be quite variable, but in no case have values below 3 mg per 100 g (fresh wt) been found in animals showing

normal blood pictures. None of the animals which died early in the study exhibited values this high (range 0.73–2.38; mean 1.35 mg per 100 g). In addition, only 2 of the remaining animals (350, 548) had values above 3.0 mg per 100 g. The lowest value was 1.01 mg per 100 g, the highest 5.4 mg per 100 g, and the mean 2.63 mg per 100 g. This was the case even though marked anemia was not present and *Haemonchus contortus* had been eliminated approximately 60 days prior to autopsy. In the case of the 2 lambs (350, 546) which received the iron supplement, the data appear contradictory. Lamb 350 exhibited a liver iron value of 4.0 mg per 100 g, while lamb 546 had only 1.01 mg per 100 g.

Chromium 51 Studies

Figure 1 illustrates the clearance curve of chromium 51-labeled red cells from

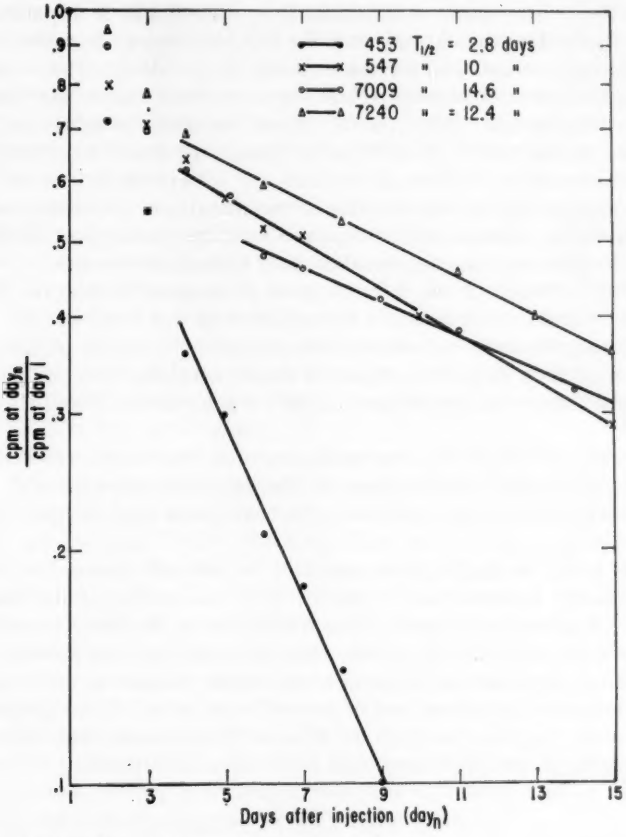

FIGURE 1. Chromium 51 clearance from peripheral blood.

the peripheral blood of 2 normal lambs (7009, 7240) and lambs 453 and 547. It is noted that in 3 of the lambs (547, 7240, 7009) that the curves consisted of at least 2 components during the first 5 days. This probably results from an inconstant elution of chromium 51 from red cells. From the 5th day on, the curves in these 3

lambs represent an exponential process. Because of this, the latter portion of the curves were extrapolated to zero time in order to determine the relative half-life of red cells. In the case of lamb 453, the clearance was rapid and consisted entirely of an exponential process. The relative half-life for red cells in the 4 lambs was as follows: 7240, 12.4 days; 7009, 14.6 days; 547, 10 days; 453, 2.8 days. Additional data on the lambs are presented individually.

Lamb 7240. In this animal, the red cells incubated with chromium 51 absorbed 76.7 percent of the isotope. By the 14th day, 70 percent of the injected chromium 51 had been excreted in urine and 3.5 percent in the feces. An additional 26.5 percent was still present in the blood.

After the 6th day, the daily fecal excretion of chromium 51 was equivalent to that contained in approximately 55 ml of blood.

Lamb 7009. The uptake of chromium 51 by the red cells of this animal was 77 percent. By the 14th day, 80 percent of the injected isotope was excreted in urine, 7.4 percent in feces, and 11.2 percent remained in the blood. This accounted for 98.6 percent of the injected material and suggested there was no appreciable tissue retention of the isotope. After the 6th day of the trial, the daily fecal excretion represented approximately 70 to 80 ml of blood. It should be pointed out that radioactive material in the feces of this lamb and 7240 probably does not represent intestinal hemorrhage, but rather is due to the excretion of the eluted isotope from red cells in the blood stream and perhaps also from the small amount of blood which normally finds its way into the intestinal tract through the mucosa.

Lamb 547. This lamb was in rather good physical condition at the time of the chromium 51 trial. At this time the hemoglobin level was 10.8 g per 100 ml and at no time during this study had anemia been present. The uptake of isotope by red cells was found to be 80 percent. It should also be noted that even though *Haemonchus* sp. was not present, the relative half-life of the red cells (fig. 1) was slightly shortened.

Lamb 453. This lamb was very small, emaciated, and showed considerable intermandibular edema at the time of the trial. During the course of the trial, the PCV dropped from 14.0 to 8.0 percent, while the hemoglobin level dropped from 4.3 to 2.2 g per 100 ml.

In this lamb, the uptake of chromium 51 by red cells was only 41.5 percent. This is probably accounted for by the low PCV (8 percent) of the diluted incubated red cell suspension. On the 4th and 10th days of the trial, an amount of isotope comparable to 250 ml and 175 ml, respectively of blood was found in the feces.

On the 9th day, 6 percent of the injected isotope remained in the blood, 36 percent had been excreted in feces, and 61 percent in the urine. This represents a total of 103 percent, but is in line with the error of the procedure and again indicates that no significant amount of chromium 51 is retained in tissues.

DISCUSSION

Since it is improbable that any of these animals escaped infection with *Haemonchus contortus,* it seems expedient to discuss the data in terms of the imposition of the pathogenesis of other species on the established pathogenic effects of this nematode. Support for this assumption is found in the work of Andrews (1942) and Stewart (1950), dealing with the relatively rapid development of resistance in sheep infected with *Haemonchus contortus.* It is not possible to state whether the

lambs which did not develop marked anemia "lost" their infection with *Haemonchus contortus* prior to developing an anemia, or rather had already suffered from anemia and then had recovered in much the same manner as lamb 476 did following treatment. The former situation is more likely since the owner would probably have noted the appearance of such severe disease prior to July if it had occurred.

Even though *Haemonchus contortus* produces disease principally by removal of the blood of its host, the peripheral blood pattern may be quite varied under different conditions. This has been discussed in a report by Whitlock (1950) in which he showed that both a microcytic, hypochromic, leptomorphic and a macrocytic, hyperchromic, normomorphic anemia could be produced in sheep with a predominant infection of *Haemonchus* or in sheep with species of *Trichostrongylus* and *Cooperia* predominating. The controlling factor was found in the dietary source of materials essential to hemopoiesis.

Peripheral blood studies from lambs in this study indicate that the condition is not always so unequivocal as in Whitlock's experimental animals. In this case, animals demonstrating both microcytic and macrocytic anemias were observed. This may have resulted from marginal dietary sources of essential nutrients. However, it was not possible to accurately study this possibility at the time of the present investigation.

That iron deficiency may occur is well established in the present report. It also appears that there is little or no interference with the mobilization of liver iron stores in haemonchosis even though other genera of nematodes are present. It is also of interest to note that although iron stores may be severely depleted, plasma iron (MIBC) levels did not decrease until the liver iron stores reached values as low as 0.7 to 2.5 mg per 100 g of fresh tissue. In addition, it is noted that a decrease in MIBC occurred simultaneously with, or only slightly prior to, a decrease in hemoglobin. This would appear to limit the value of plasma iron determinations in assessing the total body iron balances.

That anemia of the severely affected animals was not due to uncomplicated iron deficiency is indicated by the TIBC of the anemic lambs. In true iron deficiency with decreased plasma iron, it would be expected that an increase in TIBC would occur. In only one lamb (550A) was a significant increase found. The reason for this is not immediately apparent. It has been well established in this laboratory, however, that a marked decrease in serum protein occurs in such animals. This is particularly true in animals heavily parasitized by members of the genera *Ostertagia* and *Trichostrongylus*. Since transferrin is a beta-1 globulin, it is possible the animals were unable to synthesize adequate amounts of this component. In the opinion of the authors this would be more attributable to the effects of nematode genera other than *Haemonchus*, since lowered TIBC and normal or slightly elevated MIBC are observed in such infections (Baker and Douglas, 1957b; and unpublished data), while in a limited number of uncomplicated cases of haemonchosis a decrease of plasma iron has been accompanied by an increase in TIBC.

The fact that rather large and continuous oral $FeSO_4$ supplementation did not appear to increase the liver iron stores in 2 lambs, even though they were not markedly anemic and *Haemonchus contortus* was not present, might have been due to decreased absorption resulting from catarrhal enteritis produced by *Ostertagia* sp. and *Trichostrongylus* sp.

Some information with regard to the blood-sucking propensities of *Haemonchus*

contortus, Ostertagia sp. and *Trichostrongylus* sp. may be found in the data. If the normal fecal excretion of chromium 51 is taken to represent the equivalent of 65 ml of blood (mean of lambs 7009 and 7240) and subtracted from that of lamb 453, the difference is equivalent to approximately 140 ml of blood. Since this lamb would have had a blood volume of only 1000 to 1200 ml, it is apparent he was losing from 12 to 14 percent of his blood each day. This figure is in agreement with those of Andrews (1942) in lambs experimentally infected with *Haemonchus contortus.* If this blood loss is considered to be entirely due to *Haemonchus contortus,* then the average daily loss per adult parasite would have been 0.08 ml.

Since *Haemonchus contortus* was not present in any of the lambs after July 20, certain assumptions regarding the effects of the genera *Ostertagia* and *Trichostrongylus* can be made. As previously mentioned, it appears that these parasites may interfere with the absorption of iron, as well as with the TIBC of the plasma, because none of the lambs appeared to increase their liver iron stores even after a period of 60 days. Another explanation of this observation might be based on the assumption that these nematodes also remove significant amounts of blood. The data is not adequate to allow clarification of this point because, even though the relative half-life of red cells in lamb 547 was found to be decreased by approximately 20 percent, this shortening could well have been due to intravascular removal of cells as has been reported (Baker and Douglas 1957b) in chronic mixed infections with *Ostertagia* sp. and *Trichostrongylus* sp. This phenomenon was probably due to interference with intestinal absorption, since there appears to be no critical reports in the literature which indicate that *Ostertagia* sp. or *Trichostrongylus* sp. may produce hemorrhagic anemia. That intravascular hemolysis may occur in gastrointestinal parasitism of lambs has been suggested by Fraser (1930). In his report, however, he attributed this effect principally to *H. contortus,* although other species were present in rather large numbers; and he apparently did not consider gastric hemorrhage as the primary cause of anemia in *H. contortus* infection. Holman and Pattisen (1941), on the basis of Fraser's work and their own studies which indicated the anemia was generally of a hypochromic nature in gastrointestinal parasitism of lambs, concluded that the principal cause of anemia was due to interference with iron anabolism and some hemopoietic principle which occasionally produced macrocytic anemia rather than gastric hemorrhage.

No abnormalities were observed in the plasma or liver copper levels, though the possibility of underlying abnormal copper metabolism has not been entirely eliminated.

SUMMARY

Peripheral blood patterns, plasma iron components, plasma copper, and iron and copper stores of the liver were studied in 11 lambs suffering from mixed gastrointestinal parasitism as the result of infection by species of the family Trichostrongylidae.

Where the pathogenic effects of *Haemonchus contortus* predominated, a severe anemia complicated by iron deficiency was noted. In all cases the liver iron stores were reduced, but only in lambs with iron stores of from 0.7 to 2.5 mg per 100 g of fresh liver accompanied by marked anemia were plasma iron levels reduced. Dietary supplementation of $FeSO_4$ did not significantly increase the liver iron stores even though all of the *Haemonchus contortus* parasites had been removed. It is con-

cluded that this phenomenon was due either to interference with intestinal absorption or to the blood-sucking propensity of species of the genera *Ostertagia* and *Trichostrongylus.*

It was estimated that *Haemonchus contortus,* and possibly other genera, removed 140 ml, or 12 to 14 percent, of the blood of one particular lamb each day.

No changes in plasma or liver copper levels attributable to the nematodes were detected.

The peripheral blood patterns of markedly anemic animals were commensurate with that of hemorrhagic anemia with superimposed iron deficiency.

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RESEARCH NOTE

DIFFERENTIATION OF *TRYPANOSOMA RANGELI* AND *SCHIZOTRYPANUM CRUZI* IN A LIQUID MEDIUM, WITH NOTES ON THE NUTRITION OF HEMOFLAGELLATES

A relatively simple, protein-free liquid medium used in bacteriological work for the cultivation of *Hemophilus influenzae* and modified by Warren (1957, Sc.D. Dissertation, Johns Hopkins University) for the growth of *S. cruzi*, allows a clear physiological differentiation between this flagellate and *T. rangeli*. The medium is prepared by adding 10 percent of blood (frozen sheep blood) to a brain-heart infusion (Difco), heating to boiling, filtering through filter paper, and autoclaving. The clear liquid medium supports a good growth of *Schizotrypanum* (5 strains of *S. cruzi* and 1 strain of *S. vespertilionis*), yielding concentrations of about 35 million organisms per ml, but does not support growth of *T. rangeli* (2 strains).

Experiments were carried out to determine whether *S. cruzi* can produce the necessary substances for the growth of *T. rangeli*. Cultures of *S. cruzi* were set up in dialyzing bags suspended in test tubes containing the same liquid medium inoculated with *T. rangeli*. The latter species does not grow under these conditions. It was shown that the labile substances of blood required by *T. rangeli* are dialyzable. When fresh rabbit blood was added to the dialyzing bags, *T. rangeli* grew in the clear autoclaved medium outside the bag.

This medium may be useful for diagnostic differentiation after cultural isolation of the flagellates in those geographic areas where both forms of American trypanosomiasis are endemic. Furthermore, it may be used to prove the pure condition of *T. rangeli* cultures in diphasic media and for the purification of *S. cruzi* from cultures contaminated with *T. rangeli*.

We could not demonstrate any ascorbic acid in the medium by titration with 2-6-dichloroindophenol sodium salt (Harris and Ray, 1935, Lancet **228**: 71–77). On the other hand, the medium containing a known added amount of the vitamin showed the same end point, when titrated, as a standard water solution of the same concentration.

Single additions of hematin (3 mg percent), washed erythrocytes (10 percent), or horse serum (10 percent) to the brain-heart infusion liquid do not support growth of *Schizotrypanum*. Medium prepared with the addition of horse serum and hematin supports growth equivalent to that obtained in medium prepared with whole blood. Seitz-filtered cytochrome C (3–9 mg percent) does not substitute for hematin in supporting growth.

Two other hemoflagellates, *Endotrypanum schaudinni* and *Leishmania enriettii*, grow poorly in the medium and almost exclusively in the bottom of the tube, from initial populations of about 500,000 organisms per ml to about 5 to 6 million per ml. It is assumed that some of the required substances are present in very low concentrations and act as limiting factors. A strain of *T. lewisi*, maintained in a diphasic medium, did not grow in the liquid medium.

From the properties here pointed out and from the data in the literature (cf. Lwoff, M., 1951, *in* Lwoff, A., Biochemistry and Physiology of Protozoa, p. 129), it may be hypothesized that, on a nutritional basis, *Schizotrypanum* is a very primitive genus apparently closely related to *Herpetomonas* of some insects. On the other hand, the genus *Trypanosoma* contains organisms with more exacting requirements, and the genera *Endotrypanum* and *Leishmania* seem to be intermediate in phylogenetic position.

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THE EFFECTS OF REFERENCE ANTHELMINTICS AGAINST *NEMATOSPIROIDES DUBIUS* AND OXYURIDS IN MICE RELATIVE TO SCREENING PROCEDURES FOR NEW DRUGS

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This paper deals with a continuing search for adequate screening procedures that would expedite the development of better anthelmintics, particularly for combatting intestinal nematodes. Reflection upon the general problem and a review of the literature attest so clearly to the inadequacy of available methods as to require no elaboration upon the need for better methods.

Nematospiroides dubius Baylis, 1926, has been demonstrated to be infectious for laboratory mice (Baker, 1954). This contribution along with the oxyurids *Aspiculuris tetraptera* Schulz, 1924, and *Syphacia obvelata* Seurat, 1916, which occur as cosmopolitan natural infections, enables simultaneous study of drugs in mice against three intestinal nematodes that not only represent different taxonomic groups but also have predilections for different portions of the intestinal tract. *N. dubius* resides in the upper portion of the small intestine (unpublished observations) while *S. obvelata* and *A. tetraptera* have predilections, respectively, for the cecum and large intestine (Thompson and Reinertson, 1952). The effects of selected anthelmintics under various treatment regimens against these infections constitute the body of this report.

MATERIALS AND METHODS

Webster strain albino mice of either sex and of 15 to 25 g weight range served as the experimental hosts. Although they were procured from three local suppliers (Butterfield, Kalkoff, or Stout), all mice within an experiment were obtained from the same supplier.

The test helminths included the oxyurids *S. obvelata* and *A. tetraptera* and the trichostrongyle *N. dubius*. The first two were used as natural infections and *N. dubius* infections were induced experimentally.

N. dubius, kindly supplied by Dr. R. J. Shaver, Dow Chemical Co., was maintained in the following manner which differs only in detail from the procedures of Baker (1954). Strain maintenance mice that had been infected from several weeks to a few months earlier with approximately 200 larvae were placed in wire-bottom cages that were suspended over moist paper. The spillage of feed (ground Rockland mouse pellets) into the feces was prevented by means of feed hoppers. Fecal pellets were collected while still moist, and cultures were prepared by placing about 10 pellets in the center of a Petri dish lined with two layers of moist filter paper. The preparations were stored for 5 days at room temperature in a covered battery jar with open dishes of water which maintained a humid environment. Desiccation at any time proved to be very deleterious to larval development. The infective larvae, which migrated towards the edge of the filter paper, were collected by rinsing the paper with water and cleared of mouse feces by several cycles of washing and low-speed centrifugation. The larvae retained infectivity when stored at 4° to 5° C for as long as 3 weeks. Test infections were induced in mice through administration of the desired number of larvae (usually 10) by gavage in approximately 0.1 ml of water. A magnetic stirrer was used to provide a uniform suspension of larvae in the inoculum. The inoculated mice were housed for 2 weeks on wood shavings to allow maturation of *N. dubius* and to favor high oxyurid infections.

The mice were distributed randomly into an untreated control group (of 10 to 20) and into several treatment groups (of 5 to 10 each), which constituted a typical experiment.

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Drug was given by gavage, ad libitum in the previously mentioned diet, or by both methods. Gavage doses were given in 10 ml of vehicle per kg of body weight; the usual vehicle was either water or a 0.125 percent aqueous solution of hydroxyethyl cellulose. Dosages are expressed in terms of the active moiety. Controls were sham-dosed. Following treatment the mice were fasted for 24 to 30 hours and sacrificed.

Anthelmintic effects were assayed by comparing the proportions of uninfected mice and in the treated and control groups. The search for *N. dubius* entailed examination under a dissecting microscope of the entire small intestine compressed between glass plates. Pinworm examinations included search of the entire contents of the cecum and large intestine as described by Thompson and Reinertson (1952).

RESULTS

Infections in Untreated Controls. Data depicting the overall prevalence of the 3 nematodes in untreated mice serving as controls for the anthelmintic tests are presented in table I. These data are interpreted as indicative of the average prevalence

TABLE I. *Incidence of infection in control animals.*

Parasite	Supplier					
	Butterfield		Stout		Kalkoff	
	No. exam.	Pct. inf.	No. exam.	Pct. inf.	No. exam.	Pct. inf.
*S. obvelata**	1371	83.8	222	87.8	50	64
*A. tetraptera**	1371	65.5	222	92.8	50	72
*N. dubius***	1264	97.1	222	100	50	100

* Natural infections. ** Experimentally induced infections.

of infection before treatment inasmuch as they were compiled from 10 to 20 controls for each lot of mice used. For the sake of simplicity of presentation, the control data for each individual test are not given in the tabulations on drug effects, but it is to be emphasized that the computations of effects (by Abbott's formula, Abbott 1925) were based in each instance on the parallel controls.

The prevalence of the naturally occurring pinworms *S. obvelata* and *A. tetraptera* varied according to the supplier, but it would not seem appropriate to attach any significance to these differences because no attempt has been made to examine the possible influences of such variables as host size, age, sex, diet and housing during breeding, and season of the year.

The degree of regularity with which *N. dubius* was experimentally established is outstanding, particularly in view of the small number of larvae administered per mouse (usually 10 larvae and only rarely 25). Failure to induce infection was not obviously related to the number of larvae in the inocula, and it is not known whether these failures resulted from refractoriness of the mice or from the technics used.

Piperazine Hexahydrate. This reference drug was active against all 3 worms (table II). The order of decreasing susceptibility was *A. tetraptera, S. obvelata,* and *N. dubius* when treatment was either by drug-diet or multiple gavage doses. *N. dubius* was more susceptible to a particular total amount of drug when given in 1 or 2 large doses than in many small doses. The pinworms, in contrast, responded better to a lower total amount when given in many small doses than in a few large ones.

Phenothiazine. This drug was ineffective against *N. dubius* when given in either large doses by gavage, fed in the diet for 6 days, or administered by a combination of these regimens (table II). It has been shown, however, that *N. dubius* is susceptible to phenothiazine when mice are treated by drug-diet continuously for

periods of 24 to 70 days (Douglas et al, 1956; Baker et al, 1959). Large amounts were effective against both pinworms in the present study (table II; cf Thompson and Reinertson, 1952), but insufficient testing was done to permit a comparison of the effects of different regimens on pinworms.

TABLE II. *Effects of piperazine hexahydrate and phenothiazine.*

Amount mg/kg	Doses daily	Days dosed	Total dose mg/kg	Mice		Percent cleared*		
				No.	Pct. deaths	*N. dubius*	*S. obvelata*	*A. tetraptera*
Piperazine hexahydrate								
3,700 (2 pct. diet)		6	22,200	9	0	87	100	100
1,780 (1 pct. diet)		6	10,680	7	0	5	100	100
2,000	1	2	4,000	6	17	100	100	100
2,000	1	1	2,000	14	7	77	23	65
1,000	2	4	8,000	19	25	100	100	100
1,000	2	1	2,000	21	5	90	100	100
1,000	1	1	1,000	20	0	15	49	95
500	2	4	4,000	8	0	62	100	100
500	2	1	1,000	15	7	14	100	100
250	2	4	2,000	7	0	81	100	100
250	2	1	500	5	0	20	60	100
125	2	4	1,000	8	0	6	37	100
Phenothiazine								
2,770 (2 pct. diet)		6	16,620	9	22	0	100	84
{ 1,000	2	1	2,000 }	7	14	0	0	56
{ 1,300 (1 pct. diet)		6	7,800 }					
2,000	2	1	4,000	8	0	0	68	10
{ 500	2	1	1,000 }	6	0	0	0	0
{ 800 (0.5 pct. diet)		6	4,800 }					
1,250 (0.75 pct. diet)		6	7,500	6	0	0	56	78

* Computed by Abbott's formula : $\frac{\text{pct. controls infected—pct. treated infected}}{\text{pct. controls infected}} \times 100.$

Tetrachlorethylene and Carbon Tetrachloride. As may be seen in table III, these anthelmintics have activity against all 3 worms. *N. dubius* proved to be slightly more susceptible to both drugs than the 2 pinworms, particularly when the total amount of drug was given in 1 or 2 doses. This difference among worms was

TABLE III. *Effects of tetrachlorethylene and carbon tetrachloride.*

Amount mg/kg	Doses daily	Days dosed	Total dose mg/kg	Mice		Percent cleared*		
				No.	Pct. deaths	*N. dubius*	*S. obvelata*	*A. tetraptera*
Tetrachlorethylene								
2,000	2	1	4,000	32	3	69	38	49
2,000	1	2	4,000	13	0	37	13	8
1,000	2	6	12,000	7	14	33	56	0
1,000	2	4	8,000	18	11	92	58	30
500	2	4	4,000	10	0	89	54	51
250	2	4	2,000	5	0	80	100	20
125	2	4	1,000	8	0	29	68	11
Carbon tetrachloride								
2,000	2	1	4,000	32**	6	90	58	24
2,000	1	2	4,000	13	0	100	41	89
1,000	2	6	12,000	7	43	100	100	100
1,000	2	1	2,000	10	0	100	0	0
500	2	4	4,000	9	0	88	59	37
250	2	6	3,000	9	0	38	71	0
250	2	1	500	10	0	0		

* See Table II, footnotes.
** 16 of these mice were fasted during dosing and they responded similarly to the other 16 which were not fasted.

less marked when 8 or more doses were given. Drug-diet studies were not attempted because of the physical state of these drugs.

Santonin and Oil of Chenopodium. Neither of these substances had more than slight effect against any of the three worms (table IV). Santonin was tested ex-

TABLE IV. *Effects of santonin and oil of chenopodium.*

Amount mg/kg	Doses daily	Days dosed	Total dose mg/kg	Mice		Percent cleared*		
				No.	Pct. deaths	*N. dubius*	*S. obvelata*	*A. tetraptera*
Santonin								
2,048 (2 pct. diet)		6	12,240	12	67			
{ 500	2	1	1,000 }	6	17	0	0	0
{ 1,030 (0.5 pct. diet)		6	6,180 }					
500	2	4	4,000	9	89			
500	1	1	500	10	50	0	0	0
125	2	4	1,000	10	20	0	38	17
125	2	1	250	10	0	0		
Oil of chenopodium								
100	2	6	1,200	9	89			
50	2	6	600	7	0	0	59	0

* See Table II, footnotes.

haustively by both gavage and drug-diet. Oil of chenopodium was tested only at the maximum tolerable level when given in multiple gavage doses; possibly other regimens may have permitted detection of effect with this substance.

Hexylresorcinol. When tested under a wide variety of conditions, this anthelmintic showed discernible effect against *N. dubius* and *S. obvelata* but not against *A. tetraptera* (table V). The best effects with least host toxicity were seen against

TABLE V. *Effects of hexylresorcinol.*

Amount mg/kg	Doses daily	Days dosed	Total dose mg/kg	Mice		Percent cleared*		
				No.	Pct. deaths	*N. dubius*	*S. obvelata*	*A. tetraptera*
1,019** (1 pct. diet)		6	6,105	10	100			
1,030 (0.75 pct. diet)		6	6,180	7	14	0	100	0
500	2	4	4,000	10	80			
500	1	2	1,000	12	84			
500	1	1	500	10	20	0	0	0
250	2	1	500	52***	6	68	36	0
250	1	2	500	7	0	0	5	32
125	2	1	250	10	0	20	13	0
62.5	2	4	500	23	65	28	51	0
62.5	2	3	375	7	14	50	0	0
31.25	2	4	250	38	11	9	0	6

* See Table II, footnotes. ** Diet not accepted well. *** 26 of these mice were fasted during dosing and they responded similarly to non-fasted mice.

N. dubius when 2 gavage doses were given on the same day, but there was a suggestion that more effect could be achieved against *S. obvelata* by drug-diet or multiple gavage doses.

Gentian Violet, Pyrvinium Chloride and Dithiazanine Iodide. None of these substances eradicated *N. dubius* when fed for 6 days in a wide range of diet concentrations, including the highest tolerable levels. Gentian violet was also ineffective against *N. dubius* when tested (in 7 mice) by gavage in doses of 25 mg per kg twice daily for 4 days; doses of 50 mg per kg in a similar regimen were toxic. The strong antioxyurid activities of these substances by drug-diet were observed as reported elsewhere (Thompson and Reinertson, 1952; Weston et al, 1953; McCowen et al, 1957). In contrast, neither pyrvinium chloride nor gentian violet had strong antioxyurid activity when given in tolerable doses by gavage (Weston et al, 1953).

Bephenium. This new anthelmintic (cf Copp et al, 1958) was tested as the

pamoate (embonate) salt in 2 groups of 7 mice each. One group was given 2 doses of 1000 mg per kg by gavage within a 24-hour period and then was fed a 0.5 percent diet concentration for 6 days. The second group was treated similarly except that the amounts were halved. In both groups the percentage of mice infected with *N. dubius* was reduced 57 percent, but the proportion of mice infected with each pinworm was not altered.

DISCUSSION

The foregoing results have several noteworthy implications relative to screening procedures. Of foremost importance is the fact that the 3 worms together yielded gratifying qualitative information since they enabled the detection of activity in 9 of the 11 anthelmintics tested, the exceptions being santonin and oil of chenopodium. The activities of the individual drugs corresponded to previous reports on their effects against oxyurids (Thompson and Reinertson, 1952; Weston et al, 1953; and McCowen et al, 1957) and *N. dubius* (Hewitt et al, 1957). It is to be noted that neither a single worm with several treatment regimens nor all 3 worms with a single treatment regimen consistently showed the various reference drugs to be active. However, when the 3 worms are collectively considered under a variety of treatment regimens reference drug activity usually was apparent. The latter fact suggests that the initial testing of new substances of completely unknown potentialities should incorporate more than a single treatment regimen. Hence, in current work in these laboratories the initial testing of new substances involves the administration of 2 doses of drug by gavage within a 24-hour period followed by feeding of the drug in the diet for 6 days. Active substances encountered by the joint regimens are then examined further by a single regimen.

A second contribution of the present results is the provision of a background for determining whether or not a new substance has potential value in man or livestock. The important point is amply demonstrated that doses of reference drugs requisite for effect in mice are many-fold larger on a weight basis than are those recommended for use in man and large animals. It is recognized that assays based on partial rather than complete effect probably would have enabled the demonstration of activity by lower doses but complete effect criteria were selected for convenience and because they correspond to the goal of parasitologic cure. In the case of a new drug, this suggests that the amount requisite for complete effect in mice has little significance insofar as its use as an effective vermifuge in larger animals is concerned. Whether or not the drug has selectively greater toxicity for the parasite than for the host would seem to be a better criterion for estimating overall promise, but for obvious reasons even such therapeutic index criteria have only limited significance. A reliable answer as to actual value can be supplied only by tests in the final hosts themselves.

It may be useful to review some of the advantages and disadvantages of the present 3-nematode test in mice. Obvious advantages are associated with the use of readily available or easily induced infections in a small and inexpensive host that requires small drug samples, is easily housed, and can be used in large numbers. The mouse oxyurids seem to be particularly useful in screening for pinworm drugs since they are highly susceptible to the best drugs against human pinworms, namely, pyrvinium, dithiazanine, piperazine, and gentian violet. *N. dubius* is variably susceptible to most of the drugs that are highly effective against human ascarids or

hookworms, namely, piperazine, hexylresorcinol, tetrachlorethylene, carbon tetrachloride, and bephenium; but it is refractory to several others that have received varying amounts of endorsement for these purposes in man or dogs, namely, santonin, pyrvinium, dithiazanine, diethylcarbamazine (Hewitt et al, 1957) and possibly oil of chenopodium. Finally, mention should be made of the fact that unpublished work in these laboratories has shown that, with appropriate measures, testing against *Hymenolepis nana* can be done along with the above 3 nematodes so that substances can be screened simultaneously for both antinematodal and anticestodal activity.

The limitations of the present test are too numerous to list all of them in detail, but some of the more obvious defects are the lack of attached worms such as hookworms, of mucosal-dwelling worms such as *Strongyloides*, and the over-riding fact that one can rarely extrapolate with any degree of confidence from results in mice to larger animals, particularly when the same parasites are not involved.

SUMMARY

Eleven reference anthelmintics were tested against experimental *Nematospiroides dubius* infections and naturally occurring *Syphacia obvelata* and *Aspiculuris tetraptera* infections in mice. Piperazine, phenothiazine, tetrachlorethylene, carbon tetrachloride, hexylresorcinol, gentian violet, pyrvinium chloride, dithiazanine iodide, and bephenium embonate were active against one or more of the worms, but santonin and oil of chenopodium were not appreciably active.

The study was directed toward determining the susceptibility of the three test worms to representative reference drugs and the influence of treatment regimen on activity.

Inasmuch as neither gavage nor drug-diet treatment proved to be optimal for all drugs and no single worm was optimal for showing activity by all drugs, a combination of these regimens against all three worms would seem to be preferred for the adequate screening of new substances.

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PRELIMINARY ANTHELMINTIC STUDIES WITH *NEMATOSPIROIDES DUBIUS* IN MICE

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Baker (1954) described the host-parasite relationship of *Nematospiroides dubius* in mice and suggested the use of this system as an alternate means for studying the pathogenesis of the trichostrongylidoses of domestic animals. This system, moreover, seems to meet the requirements of a good laboratory evaluation test for anthelmintics as outlined by Brackett and Bliznick (1949) because uniform infections are readily reproducible in test animals, the host is small, uniform in strain, and available in large supply at low cost permitting the use of minimum quantities of drug. The evidence of drug effectiveness is easily read and the resultant activity is capable of reproduction.

Hewitt and Gumble (1957) working with *N. dubius* in mice reported that relatively large doses of 7 out of 9 standard anthelmintics were capable of significantly reducing the numbers of eggs and/or adult worms.

More recently, Copp et al (1958) reported from England on a series of chemical compounds which exhibited activity against *N. dubius* in mice. Subsequent trials in dogs, cats, and sheep confirmed the validity of the laboratory studies.

These observations indicate that the *N. dubius*-mouse relationship can be considered potentially useful for detecting anthelmintic activity under laboratory conditions.

The purpose of this report is to present data on some studies with Compound 1871. This is the first anthelmintic to be reported in this country through testing with *N. dubius* in mice.

MATERIALS AND METHODS

The structural formula of Compound 1871 (2-thienylmethyl 2-imidazolinyl sulfide hydrochloride) which is completely water soluble, is presented in figure 1. In all experiments it was administered per os in a volume of 0.2 ml per dose.

All stages of *N. dubius* were handled in a manner similar to that described by Baker (1954).

Preliminary studies were conducted to determine the minimum number of larvae required to produce a consistent, reproducible infection. Although as few as 10 larvae per mouse yielded a consistent infection, it was decided to use an inoculum of 40 larvae per mouse for primary screening, because standard anthelmintics were comparatively ineffective against this degree of larval exposure. An average of 30 adult worms in the untreated controls resulted from the inoculum (40 larvae per mouse).

Webster strain male albino mice weighing approximately 18 g at the time of inoculation were used as the test animals. Mice inoculated per os with *N. dubius* larvae were held for a period of 15 days in groups of 50 per cage.

At the time of treatment, infected mice were placed in suitable containers in groups of 10. All mice were held for 2 or 3 days after completion of treatment before sacrifice.

The small intestine of each mouse was examined at autopsy for the presence of *N. dubius*. To establish a criterion for measuring activity, only those mice in which no worms were found were considered cleared of the infection. Displacement of worms, which was observed in many instances, and reduction in ova count were not considered in measuring drug activity. The average number of worms for each treated group was compared to that shown by the untreated controls. Such a procedure permitted computation of the efficiency for each regimen studied.

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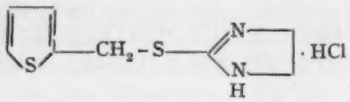
Figure 1—Compound 1871
2-thienylmethyl 2-imidazolinyl sulfide hydrochloride



$C_8H_{10}N_2S_2HCl$

RESULTS AND DISCUSSION

The results are presented in tables I and II. All multiple dose levels, except the 12.5 mg per kg level when administered for 2 doses, completely cleared a high percentage of infected mice. Some variations were noted for the different levels studied. For example, 100 mg per kg when administered for 2 doses cleared a higher percentage of mice than a comparable dosage schedule at 200 mg per kg. Such a difference might be explained by the fact that the presence of even 1 worm in a mouse eliminated the possibility that such an animal could be considered cleared of the infection. Furthermore, 3 times the number of mice were used on the 100 mg per kg level. The percent worm reduction brought about by these levels demonstrates the inconsequential significance of the variation in therapeutic activity.

TABLE I. *The activity of Compound 1871 in multiple doses against* Nematospiroides dubius.

Level mg/kg	Number of doses	No. mice autopsied	No. mice cleared	Percent cleared	Average worm burden/mouse	Percent worm reduction
200	3	22	19	86	0.3	99
200	2	32	22	69	2.3	92
100	2	95	84	88	0.6	98
50	5	10	10	100	0.0	100
50	3	49	37	75	0.7	98
50	2	43	37	86	0.7	98
25	3	39	24	61	2.8	90
25	2	30	19	63	0.9	97
12.5	3	20	13	65	1.4	95
12.5	2	20	6	30	4.1	86

Average worm burden of infected controls was 30 worms per mouse.

When the figures presented under the heading "Average Worm Burden per Mouse" are studied the real significance of the activity of Compound 1871 becomes apparent. Compared to the numbers of worms in the untreated controls, even the 12.5 mg per kg level (2 doses) exhibited an 86 percent worm reduction.

The activity of this new anthelmintic is even more striking when the data shown in table II are examined. Single doses of 300 mg per kg down to 25 mg per kg are capable of bringing about a greater than 90 percent worm reduction and completely eliminating the infection in a high percentage of the experimental animals.

TABLE II. *The activity of Compound 1871 in singles doses against* Nematospiroides dubius.

Level mg/kg	No. mice autopsied	No. mice cleared	Percent cleared	Average worm burden/mouse	Percent worm reduction
300	66	53	80	0.8	97
200	88	64	73	1.1	96
150	20	15	75	0.5	98
100	113	78	70	1.0	97
50	55	38	69	0.6	98
25	19	12	63	2.3	92
12.5	20	0	0	15.9	47

Average worm burden of infected controls was 30 worms per mouse.

For comparative purposes, single doses of phenothiazine, tetrachloroethylene, cadmium oxide, hexylresorcinol, and piperazine monohydrochloride were evaluated at levels of 300 mg per kg, 200 mg per kg, and 100 mg per kg. The results of these trials are shown in table III. Only hexylresorcinol and cadmium oxide exhibited measurable activity against the *N. dubius* infection. The activity of these compounds, however, is hardly comparable to that demonstrated by Compound 1871, especially when it is noted that comparatively few mice were completely cleared of the infection. Phenothiazine, the accepted drug of choice for the treatment of many gastro-intestinal parasites in farm animals, exhibited no measurable activity.

TABLE III. *The activity of single doses of standard anthelmintics against* Nematospiroides dubius.

Drug	Level mg/kg	No. mice autopsied	No. mice cleared	Percent cleared	Average worm burden/mouse	Percent reduction
Tetrachloroethylene	300	20	0	0	15.5	48
	200	20	0	0	22.3	26
	100	10	0	0	22.5	26
Hexylresorcinol	300	19	6	31	7.6	78
	200	20	4	20	14.5	51
	100	10	0	0	19.0	37
Phenothiazine	300	20	0	0	30.0	0
	200	10	0	0	30.0	0
	100	10	0	0	30.0	0
Cadmium Oxide	300	8	1	13	9.0	70
	200	9	3	33	13.0	57
	100	16	1	6	9.0	70
Piperazine Monohydro chloride	300	20	0	0	19.0	37
	200	20	0	0	30.0	0
	100	20	0	0	30.0	0

Since highly significant activity had been demonstrated with Compound 1871 under these conditions it seemed logical to determine what effect higher inocula would have on the activity of this new therapeutic agent. Experimentation has shown that the 100 mg per kg level (2 doses) is capable of bringing about a 95 percent worm reduction resulting from an inoculum of 500 larvae per mouse. A regimen of 50 mg per kg (2 doses) brought about a 96 percent reduction against an inoculum of 200 larvae per mouse. From these observations it can be concluded that *N. dubius* larvae when inoculated in large numbers do not appreciably decrease the activity of Compound 1871.

Subsequent reports will be presented describing the therapeutic activity of Compound 1871 against *Nippostrongylus muris,* a trichostrongyle of rats, and *Trichostrongylus axei* in rabbits.

Compound 1871 has also been found highly effective against ova and/or larvae of *N. dubius, N. muris,* and *T. axei.* The results of these studies with Compound 1871 against the intermediary stages of these parasites will be presented at a later date.

Since the *N. dubius* mouse-parasite relationship is a comparatively new biological system for detecting potentially useful anthelmintics, the true significance of its value as a screening test can only be verified by the results of evaluation tests against host-parasite systems of economic and public health importance.

Compound 1871 is being evaluated for both preventive and therapeutic activity in farm animals.

SUMMARY

The therapeutic activity of Compound 1871, a new anthelmintic, has been evaluated under laboratory conditions against *N. dubius* infection in mice. Levels as low as 12.5 mg/kg were capable of eliminating the infection. Comparisons have been

made with established anthelmintics which were found to be somewhat suppressive in effect, but which did not exhibit the order of activity shown by this new anthelmintic.

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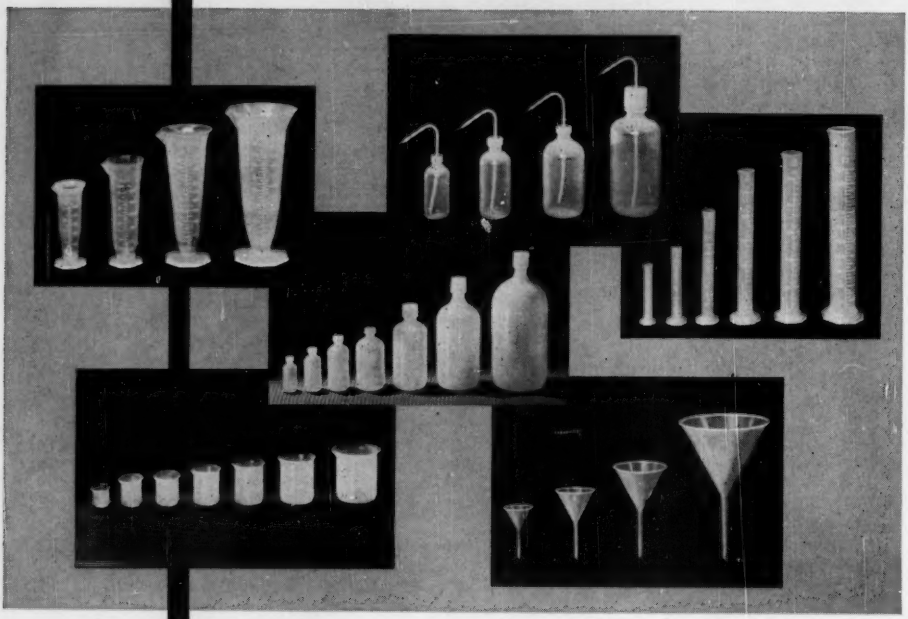

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